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(54) Title: CHIMERIC PROTEINS INCLUDING PROTEASE NEXIN-1 VARIANTS

(57) Abstract

Chimeric proteins, also referred to herein as variants, and methods of producing, formulating and utilizing such proteins are disclosed. Five different general types of variants are disclosed.

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CHIMERIC PROTEINS INCLUDING PROTEASE NEXIN-1 VARIANTS

Field of the Invention

This invention relates generally to the field of proteins, protein analogs, and methods of specifically altering proteins in order to change their biological activity. More particularly, the invention relates to identifying the active site of a protein, for example, Protease Nexin-1, and altering the active site by changing one or more amino acids therein to create an analog or replacing the active site of a protein with the active site of a completely different protein to create a chimeric protein. The invention also relates to identifying cysteine residues, or amino acid residues which may be substituted by cystine without abolishing activity, and attaching polyethylene glycol to the thio group of cysteine, thereby increasing protein stability.

Background of the Invention

It has been known for some time that proteins have specific functions in the body. For example, natural physiological functions such as tissue remodeling, inflammation, coagulation, and fibrinolysis require proteolytic enzymes. Of particular importance is a mechanistic class of proteases called serine proteases. It is also known that the active site of all functional members of the serine protease family contains a characteristic catalytic triad consisting of serine (hence the name), aspartic acid and histidine. The hydroxyl group of the catalytic site serine is involved in a nucleophilic attack on the carbonyl carbon of the peptide bond to be hydrolyzed resulting in acylation of the protease and hydrolysis of the peptide bond. This is

followed rapidly by a deacylation step resulting in the release of intact protease.

In order to provide examples of the present invention the inventors have focused on Protease nexin-1 (PN-1) which is a serine protein purified from serum-free medium conditioned by human foreskin cells (Scott, R.W. et al., J Biol Chem (1983) 58:1043910444). It is a 42 kd glycoprotein which is released by fibroblasts, myotubes, heart muscle cells, and vascular smooth muscle cells. Its release, along with that of plasminogen activator, is stimulated by phorbol esters and by mitogens (Eaton, D.L. et al., J Cell Biol (1983) 123:128). Native PN-1 is an approximately 400 amino acid protein containing about 10% carbohydrate. Since it is present only in trace levels in serum, it apparently functions at or near the surfaces of interstitial cells. PN-1 inhibits all the known activators of urokinase proenzyme, plasmin, trypsin, thrombin, and Factor Xa (Eaton, D.L. et al., J Biol Chem (1984) 259:6241). It also inhibits tissue plasminogen activator and urokinase. However, PN-1 does not inhibit elastase or cathepsin G.

In our earlier application now U. S. Patent 5,187,089 we noted that the reactive site region of PN-1 acts as a substrate analogue and postulated that it might be possible to drastically alter PN-1 activity by modifying the reactive site sequence of PN-1, thus changing its protease specificity. Similar efforts with α-1-antitrypsin, for example, resulted in variants with altered and therapeutic potential (M. Courtney et al., Nature (1985) 313:149-151). PN-1 is different from most serpins in that it is found in tissues, contains a high affinity heparin binding site which localizes it to tissues, and has a tissue clearance receptor that is responsible for endocytosis of protease-PN-1 complexes. We were able to generate PN-1 variants as inhibitors of

physiologic proteases such as elastase and thereby provide useful pharmaceutically active compounds.

We have now invented a number of additional PN-1 variants, including variants which inactivate elastase and cathepsin G, and have moved well beyond our prior work to provide variants and methods for designing and producing such variants which have significantly altered protease specificity and second-order association rate constants with respect to a variety of serine proteases. In addition, we provide variants and methods of producing such variants which have polyethylene glycol specifically attached to one or more cysteine residues, such cysteine residues being either present in the parent molecule or introduced on the surface of the protein by site-directed mutagenesis, and methods for determining appropriate sites for the introduction of cysteine residues. In addition, we provide variants and methods of producing such variants which combine the specific localization ability of a receptor-binding protein with the protease-inhibiting activity of PN-1 or variant thereof, resulting in desired biological activities with particular substrates.

Summary of the Invention

Chimeric proteins, also referred to herein as variants, and methods of producing, formulating and utilizing such proteins are disclosed. Five different general types of variants are disclosed. A Type I variant of the invention is produced by site-directed mutagenesis wherein a single amino acid within the active site of PN-1 is substituted with an amino acid different from the naturally-occurring amino acid at that position. A Type II variant of the invention is similar to a Type I variant in that the active site of PN-1 is changed. However, to produce a Type II variant, the active site of

PN-1 is changed so as to match the active site of another serpin which change many require one or more amino acid substitutions, deletions or additions. A Type III variant of the invention is produced whereby the active site or a portion thereof of PN-1 is substituted with a sequence which corresponds to the substrate sequence for a particular protease. A Type IV variant is produced wherein a cysteine residue, which is either present in the native protein or introduced by site-specific mutation, is used to attach polyethylene glycol. A Type V variant of the invention involves producing a fusion protein wherein PN-1 is fused to the receptor binding region of another protein in order to localize PN-1 to a different receptor. Variants of Type I, II or III are referred to herein as variants. The compounds of Type IV wherein polyethylene glycol is attached to a thio group are referred to as cysteine-PEGylation proteins, and the compounds of Type V are referred to as fusion proteins or chimeric proteins.

Embodiments of the present invention provide pharmaceutical compositions which contain one or more variants of all or any of Type I, II, III, IV or V.

An important object of the invention is to provide a wide range of different variants, and in particular PN-1-variants, having a particular and desired biological activity.

Another object is to provide PN-1 variants using site-directed mutagenesis in order to change a single amino acid within the active site of PN-1.

Another important object is to provide a PN-1-variant wherein the active site of PN-1 is specifically modified so as to match the active site of another enzyme inhibitor, preferably another serpin.

Yet another important object of the present invention is to provide variants such as protease nexin-1

variants which include, in PN-1, the substrate sequence for a different protease, making it possible to inhibit the activity of that protease.

Still another important object is to provide proteins which are PEGylated by attachment to a thio group, i.e. the polyethylene glycol is attached to a cysteine amino acid within a protein, which cysteine amino acid of the protein is not involved in a disulfide bond.

Another important object is to provide a method of attaching polyethylene glycol to a protein by first subjecting the protein to site-directed mutagenesis to add a cysteine residue at a position where the protein or a structurally related protein is normally glycosylated, and thereafter attaching the polyethylene glycol to the cysteine residue.

Another important object is to provide a method of attaching PEG to a protein by first subjecting the protein to site-directed mutagenesis to add a cysteine residue at a position on the surface of the protein, and thereafter attaching the PEG to the cysteine residue.

Another important object is to provide dimeric or multimeric proteins cross-linked by reaction with a reagent composed of PEG having two protein-reactive moieties.

Yet another important object is to provide fusion proteins wherein the receptor binding region of another protein is connected to PN-1 in order to localize PN-1 to a different receptor.

Another object of the present invention is to provide a pharmaceutical composition comprising excipient carrier materials having a compound of the invention dispersed therein.

Another object of the present invention is to provide therapeutic methods of treatment which involve

administering to a patient in need thereof a pharmaceutically effective amount of a composition comprising excipients and a compound of the invention.

A feature of the present invention is that the variants can be designed to have a specific receptor-binding domain while maintaining the natural biological activity of the protein to which the new binding domain is attached.

An advantage of the present invention is that the variants have substantially different inhibitory effects on certain proteolytic enzymes as compared to the natural protein.

Another object of the present invention is to provide variants useful in treating diseases associated with a specific biological activity.

Yet another advantage of the present invention is to describe and disclose variants which are useful in treating elastase-related diseases.

Another feature of the present invention is that certain variants have substantially altered protease specificity as compared with the natural protein.

Another advantage of the present invention is that certain variants have substantially greater second order association rate constants with respect to particular serine proteases as compared with the second order association rate constant of natural proteins with respect to such serine proteases.

Another advantage of the present invention is that certain variants have substantially slower second order association rate constants with respect to particular serine proteases as compared with the second order association rate constant of natural proteins with respect to such serine proteases.

Yet another object is to provide methods of delivery such as by injection, internasal and

interpulmonary delivery which methods are carried out using pharmaceutical compositions in the form of injectable formulations, spray formulations and aerosols.

Another advantage is that biologically stabilized proteins can be produced by attaching the polyethylene glycol to a cysteine residue of the protein.

Another advantage is to provide methodology for readily attaching polyethylene glycol molecules to proteins at a cysteine residue of the protein which are preferably located at native sites of glycosylation.

Yet another advantage is that amino acid residues for substitution with cysteine may be selected so that subsequent attachment of polyethylene glycol to the thio group of the substituted cysteine residue increases biological stability of the cysteine-PEGylated protein relative to wild type without abolishing biological activity.

Another advantage is that proteins which normally require glycosylation for biological stability may be produced commercially by expression in a prokaryotic host or other host which does not provide for glycosylated recombinant proteins. After expression of the protein by the prokaryotic host, biological stability of the protein can be increased by attachment of polyethylene glycol to a native or engineered cysteine residue in the protein.

Another advantage is that cysteine-PEGylated proteins can be produced without exposing the protein to highly toxic chemicals such as dioxane, cyanuric chloride, DMF, or other chemicals used in conventional methods for attaching polyethylene glycol to a protein.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the structure, synthesis, formulation and usage as more

fully set forth below reference being made to the accompany figures forming a part hereof.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of the coding region and the deduced amino acid sequence of PN-1α; and

Figure 2 shows the nucleotide sequence of the coding region and the deduced amino acid sequence of PN-1β.

Figure 3 is a schematic drawing of a three-dimensional structure of PN-1 as determined by X-ray crystallography. The approximate position of residues of particular interest are shown according to their relative position within a given helix (h) or β-sheet (s). The helices and β-sheets of the PN-1 protein are each assigned letters (e.g. A, B, etc.) (Engh, et al. 1990 *Protein Engin.* 3(6):469-477).

Figure 4 is a graph which shows the activity of samples of reaction mixtures containing cysteine-PEGylated PN-1 variants (N99C;N140C) produced by the method of the invention (open squares) and the activity of samples of reaction mixtures containing a PEGylated PN-1 variant (N99C;N140C) produced by a conventional method (closed diamonds).

Detailed Description of the Preferred Embodiment

Before the present compounds, variants, formulations and methods for making and using such are described, it is to be understood that this invention is not limited to the particular compounds, variants, formulations or methods described, as such variants, formulations and methodologies may, of course, vary. It is to be understood that the terminology used herein is for the purpose of describing particular embodiments

only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It must be noted that as used in the specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a protease nexin-1 variant" includes mixtures of such variants, reference to "an analog" includes reference to mixtures of such analogs and reference to "the method of treatment" includes reference to one or more methods of treatment of the type which will be known to those skilled in the art or will become known to them upon reading this specification, and so forth.

A. Definitions

As used herein, "protease nexin-1" and "PN-1" are used interchangeably and refer to DNA codons and resulting amino acid sequences which make up PN-1α and PN-1β which are shown respectively in Figures 1 and 2. PN-1 is distinguishable from the two other protease nexin factors, PN-II and PN-III (Knauer, D.J. et al., J Biol Chem (1982) 257:15098-15104), which are also thrombin inhibitors, but are less strongly binding to this protease and are of different molecular weight, three-dimensional structure and mechanism of function.

The terms "variants", "protein variants" and "chimeric proteins" are used interchangeably herein to refer to any amino acid sequence which corresponds to the amino acid sequence of a natural protein or a biologically active portion of a natural protein except that some change has been made in the structures. Typical changes per the present invention involve: (1) one or more amino acids within the natural sequence is replaced with one or more amino acids different from

the amino acids present in the natural protein; and/or (2) one or more amino acids has been added to the natural sequence, and the addition of such amino acids changes the biological activity of the variant; and/or (3) one or more amino acids is deleted from the natural sequence; and/or (4) polyethylene glycol is bound to a thio group of a natural or artificially introduced cysteine residue of a sequence; and/or (5) two naturally occurring sequences are fused together, i.e. two sequences not normally connected are fused.

"Protease nexin-1 variants" and "analogs of protease nexin-1" are terms which are used synonymously herein to define a Type I variant and are thereby encompassed by the term "variant." The terms are intended to refer generally to proteins wherein one or more of the amino acids within protease nexin-1 have been substituted with a different amino acid. More specifically, the protease nexin-1 variants of the invention include substantially the same amino acid sequence as protease nexin-1 but for the substitution of different amino acids at or near the active site. Specifically, substitutions of different amino acids can be made at any of P_1, P_2, P_3, P_4 sites and/or made at the P_1', P_2', or P_3', P_4' sites. Although other substitutions and deletions of amino acids in the sequence of protease nexin-1 are encompassed by this invention, the substitutions at or near the active site are most important with respect to changing the specificity and/or reactivity of the variant with respect to particular proteases. Particularly preferred protease nexin-1 variants of the invention are variants which have high activity relative to a substrate to which natural PN-1 has little or no activity such as variants which inhibit elastase and, more particularly, which inhibit elastase and have their ability to inhibit elastase enhanced in

the presence of heparin and/or heparin-like compounds. Other preferred protease nexin-1 variants, for example, have increased ability to inhibit urokinase and/or another serine protease as compared with protease nexin-1.

"Control sequence" refers to a DNA sequence or sequences which are capable, when properly ligated to a desired coding sequence, of effecting its expression in hosts compatible with such sequences. Such control sequences include at least promoters in both procaryotic and eucaryotic hosts, and preferably, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be identified. As used herein, "control sequences" simply refers to whatever DNA sequence may be required to effect expression in the particular host used.

"Cells" or "cell cultures" or "recombinant host cells" or "host cells" are often used interchangeably as will be clear from the context. These terms include the immediate subject cell, and, of course, the progeny thereof. It is understood that not all progeny are exactly identical to the parental cell, due to chance mutations or differences in environment. However, such altered progeny are included in these terms, so long as the progeny retain the characteristics relevant to those conferred on the originally transformed cell. In the present case, for example, such a characteristic might be the ability to produce recombinant PN-1.

"Purified" or "pure" refers to material which is free from substances which normally accompany it as found in its native state. Thus "pure" PN-1-encoding DNA refers to DNA which is found in isolation from its native environment and free of association with DNAs encoding other proteins normally produced by cells natively producing PN-1. "Pure" PN-1 refers to PN-1 which does

not contain materials normally associated with its _in situ_ environment in human or other mammalian tissue. Of course, "pure" PN-1 may include materials in covalent association with it, such as glycoside residues or materials introduced for, for example, formulation as a therapeutic. The term "pure" also includes variants wherein compounds such as polyethylene glycol, Biotin or other moieties are bound to the variant in order to allow for the attachment of other compounds and/or provide for formulations useful in therapeutic treatment or diagnostic procedures. "Pure" simply designates a situation wherein the substance referred to is, or has been, isolated from its native environment and materials which normally accompany it. Of course, the DNA claimed herein as purified and free of substances normally accompanying it, but encoding PN-1, can include additional sequence at the 5' and/or 3' end of the coding sequence which might result, for example, from reverse transcription of the noncoding portions of the message when the DNA is derived from a cDNA library or might include the reverse transcript for the signal sequence as well as the mature protein encoding sequence.

"Degenerate with", as referred to a DNA sequence, refers to nucleotide sequences encoding the same amino acid sequence as that referenced.

"Operably linked" refers to a juxtaposition wherein the components are configured so as to perform a desired function such as their natural biochemical function. Thus, control sequences or promoters operably linked to a coding sequence are capable of effecting the expression of the coding sequence.

"Heparin", "heparan sulfate" and "heparin-like compounds" are terms which are used synonymously herein. Each of the terms singly or in combination with the others is intended to encompass a large group of

compounds which are generally described as sulfated polysaccharides, which includes proteoglycans and glycosaminoglycans (GAG) which are alternating copolymers of a hexosamine and an aldouronic acid. These copolymers are found in sulfated forms and are synthesized as proteoglycans and are collectively referred to as mucopolysaccharides. Other compounds such as dextran sulfate are considered "heparin-like" for purposes of the invention. Similar alternating copolymers, especially those which are highly sulfated and thus very negatively charged, are useful "heparin-like" compounds in this invention. Extensive information with respect to "heparin", "heparin-like compounds" such as glycosaminoglycans are described in detail by Benito Casu, "Structure and Biological Activity of Heparin", published in Advances in Carbohydrate Chemistry and Biochemistry, Vol. 43, pp. 51-134, which is incorporated herein by reference to disclose such compounds which might be useful in combination with certain PN-1 variants disclosed herein.

A description of the invention is facilitated by listing the relationship between the one-letter symbols and the three-letter abbreviations for amino acids as follows:

One-Letter Symbols		Three-Letter Abbreviations
A	alanine	ala
C	cysteine	cys
D	aspartic acid	asp
E	glutamic acid	gln
F	phenylalanine	phe
G	glycine	gly
H	histidine	his
I	isoleucine	ile
K	lysine	lys
L	leucine	leu
M	methionine	met
N	asparagine	asn
P	proline	pro
Q	glutamine	gln
R	arginine	arg
S	serine	ser
T	threonine	thr
V	valine	val
W	tryptophan	trp
Y	tyrosine	tyr

Amino acids have the following general structural formula

$$\begin{array}{c} NH_{3+} \\ | \\ {}^{-}OOC\!-\!C\!-\!R \\ | \\ H \end{array}$$

and are classified based on the chemical composition of the "R" group as follows:

1. Aliphatic
2. Hydroxyl
3. Sulfur
4. Aromatic
5. Acidic (and amides)
6. Basic
7. Imino

Naturally occurring amino acids can be generally classified as being polar or non-polar as follows:

Polar	S, T, C, Y, D, N, E, Q, R, H, K
Non-polar	G, A, V, L, I, M, F, W, P

It is the "R" group which determines whether the amino acid will be polar or non-polar.

Amino acid residues can be generally subclassified into four major subclasses as follows:

Acidic: The residue has a negative charge due to loss of H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

Basic: The residue has a positive charge due to association with H ion at physiological pH and the residue is attracted by aqueous solution so as to seek the surface positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium at physiological pH.

Neutral/non-polar: The residues are not charged at physiological pH and the residue is repelled by aqueous solution so as to seek the inner positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium. These residues are also designated "hydrophobic" herein.

Neutral/polar: The residues are not charged at physiological pH, but the residue is attracted by aqueous solution so as to seek the outer positions in the conformation of a peptide in which it is contained when the peptide is in aqueous medium.

It is understood, of course, that in a statistical collection of individual residue molecules some molecules will be charged, and some not, and there will be an attraction for or repulsion from an aqueous medium to a greater or lesser extent. To fit the definition of "charged", a significant percentage (at least approximately 25%) of the individual molecules are charged at physiological pH. The degree of attraction or repulsion required for classification as polar or non-polar is arbitrary, and, therefore, amino acids specifically contemplated by the invention have been specifically classified as one or the other. Most amino acids not specifically named can be classified on the basis of known behavior.

Amino acid residues can be further subclassified as cyclic or noncyclic, and aromatic or nonaromatic, self-explanatory classifications with respect to the side chain substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of 4 carbon atoms or less, inclusive of the carboxyl carbon. Small residues are, of course, always nonaromatic.

For the naturally occurring protein amino acids, subclassification according to the foregoing scheme is as follows:

Acidic: Aspartic acid and Glutamic acid;

Basic/noncyclic: Arginine, Lysine;

Basic/cyclic: Histidine;

Neutral/polar/small: Threonine, Serine and Cysteine;

Neutral/polar/large/nonaromatic: Threonine, Asparagine, Glutamine;

Neutral/polar/large/aromatic: Tyrosine;

Neutral/non-polar/small: Alanine;

Neutral/non-polar/large/nonaromatic: Valine, Isoleucine, Leucine, Methionine;

Neutral/non-polar/large/aromatic: Phenylalanine and Tryptophan.

Proline

The gene-encoded amino acid proline, although technically within the group neutral/non-polar/large/cyclic and nonaromatic, is a special case due to its known effects on the secondary conformation of peptide chains, and is not, therefore, included in this defined group, but is included as a group of its own.

Other amino acid substitutions for those encoded in the gene can also be included in peptide compounds

within the scope of the invention and can be classified within this general scheme.

Variants of the invention may include commonly encountered amino acids, which are not encoded by the genetic code, for example, β-alanine (β-ala), or other omega-amino acids, such as 3-amino propionic, 4-amino butyric and so forth, α-aminoisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), cysteic acid (Cya) and methionine sulfoxide (MSO). These also fall conveniently into particular categories.

Based on the above definition,

Sar and β-ala are neutral/non-polar/small;

t-BuA, t-BuG, N-MeIle, Nle and Cha are neutral/non-polar/large/nonaromatic;

Orn is basic/noncyclic;

Cya is acidic;

Cit, Acetyl Lys, and MSO are neutral/polar/large/nonaromatic; and

Phg is neutral/non-polar/large/aromatic.

Both L and D isomers of amino acids encoded by the genetic code or otherwise are included as amino acids useful in this invention provided the resulting protein processes the required activity.

The various omega-amino acids are classified according to size as neutral/non-polar/small (β-ala, i.e., 3-aminopropionic, 4-aminobutyric) or large (all others).

The nomenclature used to describe compounds of the present invention follows the conventional practice wherein the amino group is assumed to the left and the carboxyl group to the right of each amino acid in the peptide. In the formulas representing selected specific

embodiments of the present invention, the amino- and carboxyl-terminal groups, although often not specifically shown, will be understood to be in the form they would assume at physiological pH values, unless otherwise specified. Thus, the N-terminal H^{+}_{2} and C-terminal-O^{-} at physiological pH are understood to be present though not necessarily specified and shown, either in specific examples or in generic formulas.

Serine Proteases and their Inhibitors (Serpins)

Although originally named for their mechanism of action, members of the serine protease family also show significant sequence and structural homology. Some serine proteases are very specific, cleaving only certain peptide bonds of a specific target protein while others are very nonspecific, degrading multiple target proteins into small peptides.

Serine proteases are regulated at many levels. Some are synthesized as inactive proenzymes and are activated only during specific events and at specific locations. This allows the body to respond rapidly to a physiological perturbation by activating an already present reservoir of proteolytic activity. Coagulation, for example, is carried out when circulating proenzymes such as Factor X and prothrombin are sequentially activated in response to injury resulting in a cascade of clotting activity. In addition, proteolytic activity is often localized to specific sites, such as receptor binding sites which can cause high local concentrations of protease or proenzyme ready for activation.

Once activated, it is extremely important that proteolytic activity be confined both spatially and temporally. This control is often achieved by the presence of specific inhibitors which block proteolytic activity. An important family of related proteins, the

serine protease inhibitors, or "serpins", are key in the regulation of serine proteases. Like the serine proteases, serpins were first defined by their common mechanism of action but later turned out to be highly homologous both in terms of sequence and structure.

Serpins all contain an inhibitor domain with a reactive peptide bond defined on either side by the variables P_1 and P_1'. In a direction to the left away from the reactive site, the amino acids are referred to as P_1, P_2, P_3, etc., and in a direction to the right away from the reactive site they are referred to as P_1', P_2', P_3', etc. The P_1 residue is recognized by the substrate binding pocket of the target protease which attacks the reactive peptide bond as though a normal substrate. However, hydrolysis of the peptide bond and release of the protease does not proceed to completion. The normal deacylation step is so slow that the reaction becomes essentially irreversible and the protease becomes trapped in a stable, equimolar complex.

Protease nexin-1 (PN-1) is a member of the serpin family. PN-1 is produced by many different cell types including fibroblasts, glial cells, platelets and microphages. PN-1 is secreted by cells into the extracellular environment where it binds to and inhibits target serine proteases. PN-1-protease complexes then bind back to specific cell surface receptors where they are internalized and degraded.

PN-1 is very similar, both structurally and functionally to antithrombin (AT-III). AT-III is the primary plasma inhibitor of blood coagulation. The inhibition of thrombin by AT-III in plasma is normally very weak but is accelerated significantly by the presence of heparin or by other mucopolysaccharides on the endothelial lining of blood vessels. The therapeutic value of heparin as a blood "thinning" agent is due to

its enhancement of AT-III activity. Like AT-III, PN-1 has a high affinity heparin binding site and inhibits thrombin much more rapidly (50-100 fold) in the presence of heparin. Thus PN-1 has therapeutic potential as an anticoagulant.

On the other hand, PN-1 differs from AT-III in a number of ways. Unlike AT-III, PN-1 is also a good inhibitor of the fibrinolytic enzymes urokinase and plasmin, as well as trypsin. Furthermore, PN-1 is not found in significant quantities in plasma and may function primarily in the tissues. The high affinity heparin binding site of PN-1 serves to localize it to connective tissues and cells which contain sulfated proteoglycans on their surface and surrounding extracellular matrix. Thus PN-1's primary role seems to be in regulating proteolytic activity in tissues as opposed to blood. Further evidence for the role of PN-1 is found by the fact that it is present in brain tissue and may be involved in peripheral nerve regeneration and neurite extension.

The relative efficiency with which PN-1 inhibits serine proteases can be measured by the second order association rate constant (k_{ass}) as previously described in Bieth, J.G. (Bull. Euro. Physiopath. Resp. (1980) 16:183-195), and reported by Scott et al. (J. Biol. Chem. (1985) 260:7029-7034), both of which are incorporated herein by reference to disclose and explain the meaning of the rate association constant. In general, a value for k_{ass} equal to or greater than $1 \times 10^5\ M^{-1}S^{-1}$ for a particular protease-inhibitor reaction is considered to be physiologically significant (Travis and Salveson Ann. Rev. Biochem. (1983) 52:655-709). The k_{ass} or rate association constant has inverse-mole-seconds as its units, and the larger the k_{ass}, the more rapid the inhibition. Accordingly, a k_{ass} value is always given as a

value with respect to a particular enzyme and is zero if there is no inhibition of the enzyme.

Many physiologically important protease inhibitor reactions such as elastase-α-1 antitrypsin and plasmin-α-2-antiplasmin occur with rate constants as high as 1 x 10^7 $M^{-1}S^{-1}$ or greater. The thrombin-PN-1 reaction occurs at a similar high rate in the presence of heparin.

Description of PN-1 (α and β)

Figures 1 and 2, respectively, show the amino acid sequence of PN-1α and PN-1β. The α and β forms differ by the substitution of thr_{310}-gly_{311} in PN-1β for arg_{310} in PN-1α. Alignment of the reactive site center of PN-1 with other serpins, such as antithrombin III, predicts that arginine 345 (346 for PN-1β) is the reactive site center or "P_1" site. The "P_1" site (arginine at position 345 for PN-1α and 346 for PN-1β) has been confirmed by sequencing of the peptide fragment released from PN-1 upon dissociation of complexes with thrombin. Furthermore, PN-1 normally inhibits only enzymes which cleave at arginine (the P_1 residue), such as thrombin, plasmin, trypsin, plasminogen activators, and plasma kallikrein.

Based on the above and by referring to the sequences of PN-1α and PN-1β shown in Figures 1 and 2 respectively, it can be seen that the "P_1'"site is serine at position 346 for PN-1α and serine at position 347 for PN-1β.

Description of Protease Inhibitor Action

In order to allow the body to respond rapidly, several serine proteases are synthesized at relatively high levels in their inactive proenzyme forms and are only activated during specific events. For example, coagulation is carried out when circulating proenzymes

such as Factor X and prothrombin are sequentially activated in response to an injury. This activation results in a cascade of clotting activity. Proteolytic activity is often localized to specific sites such as receptor binding sites. Once a proteolytic enzyme is activated, it is extremely important that the enzyme activity be confined both spatially and temporally. Such confinement is in part brought about by the inhibitory effect of serpins.

All serpins contain an inhibitor domain with a reactive peptide bond defined on either side by P_1 and P_1' residues. The P_1 residue (such as arginine at position 345 for PN-1α and 346 for PN-1β) is recognized by the substrate binding pocket of the target protease. Upon recognition of the "reactive" site (of the inhibitor by the protease) the protease attacks the reactive peptide bond of the inhibitor as if it were a normal substrate. However, in the case of serpin hydrolysis of the peptide bond and release of the protease does not proceed to completion. The normal deacylation step is so slow that the reaction becomes essentially irreversible and the protease becomes trapped with the inhibitors in a stable, covalent, equal molar complex. Since the P_1 residue is the predominant determinant residue recognized by the substrate binding pocket of the target protease, alteration of this residue can alter the protease specificity of the inhibitor entirely or substantially change the degree of the inhibitory effect obtainable. Residues near the P_1 residue (i.e., P_4-P_4') also contribute to protease specificity. Accordingly, alteration of these residues can also lead to modified inhibitory effects.

Variants in General

Amino acid sequences, active sites and biochemical activity of a number of natural proteins and in particular natural serpins are known. It is also known that some proteins have a high degree of activity with respect to a certain protease, whereas another protein will have virtually no activity with respect to that protease. The present inventors noted this information and deduced that it might be possible to change the protease inhibitory activity of a particular protein, in a directed manner, by replacing its active site with the active site of another protein having a completely different activity with respect to that protease. Alternatively, one can create a variant of the invention by attaching to a first protein, the receptor binding region of a second protein. In that the biochemical function and protein binding specificity of different proteins are known, the methodology of the present invention makes it possible to make certain logical deductions and create a specific variant with a relatively high expectation of not only changing the activity (e.g. binding affinity) of the protein, but changing it in a specific and directed manner.

An example of producing chimeras (Type V variants) of the invention can be carried out as follows. A first protein might be known to have virtually no binding affinity with respect to a given receptor and a second protein might have very high binding affinity with respect to that receptor. (A receptor can be any protein or a portion thereof, ligand, cell surface area or molecule.) By attaching the binding region of the second protein to the first protein one can provide a variant with the biochemical functions of the first protein which will bind to the receptor previously bound only by the second protein. A specific example of utilizing the

methodology of the present invention in order to take advantage of the binding specificity and biochemical activity of two different proteins is described below.

A specific situation to which variants of the invention might be applied is as follows. The movement of monocytes and neutrophils from the bloodstream into inflamed tissue requires activation events at the site of interaction. These events include expression of adhesion molecules and recruitment of transmigration machinery. To migrate through the endothelium, the connections between the cells must be degraded, including the basement membrane and extracellular matrix. It is now clearly established that the urokinase-type plasminogen activator (uPA)-plasmin system plays a major role in regulating extracellular proteolysis i.e. uPA-plasmin is important in breaking intracellular connections so that neutrophils can migrate through the endothelium blood vessel lining into surrounding tissues. Although the migration of some neutrophils cells through the endothelium to the surrounding tissue is desirable, too much migration too quickly results in undesirable inflammation of the tissue and, possibly, blockage of the bloodstream passing through the inflamed tissues. A variant which would (1) bind to activated endothelium cells and (2) inhibit uPA-plasmin would be useful in preventing and/or reducing inflammation at a particular site.

Tumor cell invasiveness is also dependent upon the uPA-plasmin system as shown in tumor cell metastasis model systems (Ossowski and Reich, 1983; Hearing, et al., 1988) and extracellular matrix degradation and basement membrane invasion (Gergman, et al., 1986; Mignatti, et al., 1986; Reich, et al., 1988; Cajot, et al, 1990). The levels of uPA are significantly higher in human breast cancer tissues than in normal tissues. Increased amounts

of uPAR correlate with increased invasiveness of malignant cells in model systems (Ossowski, 1988; Hearing, et al., 1988). Agents which block the proteolytic activity of uPA or plasmin may protect against extracellular matrix degradation and basement membrane invasion and aid in preventing inflammation. Similarly, agents which block the interaction of urokinase with the urokinase receptor (uPAR) might block metastasis.

A variant protein of the present invention could be designed to block the urokinase receptor and inhibit urokinase. Specifically, a variant could be produced which combined the ability to block the urokinase receptor with the ability to inhibit urokinase and plasmin, and thereby have an effect on alleviating or preventing inflammation. Such a variant would be more effective in reducing or preventing inflammation than would either protein by itself. By making use of the urokinase receptor binding ability of the variant, the variant will localize to the desired specific site of extracellular matrix degradation, specifically preventing further degradation by inhibiting enzymes and preventing the binding of enzymes which cause degradation, thereby having a dual effect on alleviating or preventing inflammation.

To produce a chimeric protein of the present invention the central role of uPA and uPAR in cancer invasion and inflammation were recognized. With this information in mind it was understood that the present invention should provide a chimeric protein which would interfere with the binding of uPA to uPAR and inhibit both uPA and plasmin generated at sites of cellular invasion.

The receptor binding region of uPA has been localized to the 135 residue amino-terminal fragment

(ATF). This region (i.e. ATF) binds to the uPAR with high affinity (K_d 0.1 to 1 nM) and can competitively inhibit the binding of uPA to the uPAR.

PN-1 blocks tumor cell-mediated extracellular matrix degradation and tumor cell migration *in vitro*, in that PN-1 inhibits the uPA-plasmin system. In order to generate a more effective and specific inhibitor of tumor metastasis and leukocyte invasion, the present invention provides a chimeric protein consisting of the amino-terminal fragment of uPA and PN-1 (ATF-PN1). Due to the presence of the ATF portion, this chimeric protein has a high affinity to sites of cellular invasion; and due to the PN-1 protein it inhibits uPA and plasmin.

Type I Variant

This aspect of the invention involves the manipulation of the amino acid sequence of the PN-1, so that the reactive site is in some way altered, to change the protease specificity or the degree of inhibitory effect of PN-1 on serine proteases. More specially, the present invention involves substituting one or more amino acids within protease nexin-1 and/or deleting or adding amino acids to the sequence of protease nexin-1 in order to obtain an effect on the reactive site of protease nexin-1 so that the protease specificity of protease nexin-1 and/or the degree of inhibitory effect of protease nexin-1 on a serine protease is changed. In general, the change in protease specificity or degree of inhibitory effect is obtained by substituting an amino acid at the P_1, P_2, P_3, P_4 or, alternatively, P_1', P_2', P_3', P_4' sites. Still more specifically, the invention involves substituting one or both of the "P_1" site arginine residue or "P_1'" site serine residue with a different residue resulting in PN-1 variants with

radically different protease specificities and/or inhibitory effects on particular serine proteases.

The PN-1 variants of the invention can also be described in terms of their functionality. Importantly, some of the PN-1 variants of the invention are capable of inhibiting elastase. Within this general group are PN-1 variants wherein the ability to inhibit elastase is greatly enhanced in the presence of heparin and/or heparin-like compounds. Another group of PN-1 variants of the invention include PN-1 variants which have an enhanced ability to inhibit serine proteases as compared with PN-1. The PN-1 variants of the invention are designed to inhibit serine proteases such as urokinase, Factor Xa plasmin, kallikrein, Factor XIIa, Factor XIa, Factor Va, tPA, elastase, cathepsin and contrapsin. Functional objectives of the invention, such as the production of a compounds which inhibit serine proteases such as elastase and whose ability to inhibit such is enhanced in the presence of other compounds such as heparin, are obtained, in general, by manipulating DNA. Specifically, the DNA encoding an enzyme is manipulated by including within the DNA a sequence of DNA which encodes a substrate for a particular serine protease. The recombinant DNA is then expressed to produce a variant of the invention which will include a substrate for the particular serine protease. In that the substrate is present, the activity of that serine protease can be specifically inhibited by the variant while the variant maintains its natural biological activity.

The five different types of variants of the invention will now be described in further detail.

PN-1 Variants -- Active Site Manipulation

For purposes of clarity, substitution at a single site will be discussed first (P_1 site then P_1' site) followed by a discussion of multiple substitutions.

Type I Variants: Single Site Mutations

The arginine residue is a polar, basic amino acid. Substitution of the polar arginine with a non-polar residue has a dramatic effect on the degree of serine protease inhibition obtainable. As a specific example of this effect, reference is made to our compound NCY2010 wherein arginine (at position 345 for α, 346 for β) is substituted with isoleucine (R345I). The R345I variant (i.e., isoleucine has been substituted for arginine a position 345 of PN-1α) essentially eliminates thrombin inhibitory activity with or without heparin. At the same time, the NCY2010 variant is a good inhibitor of neutrophil elastase activity. This is surprising when it is noted that native fibroblast PN-1 has no inhibitory effect on elastase, and in fact is a substrate for elastase.

The activity of NCY2010 should be viewed with the understanding that the relative efficiency at which protease inhibitors (such as the PN-1 variants of the invention) inhibit serine proteases are measured by a known standard. That standard is the second order association rate constant (k_{ass}) as described in (Bieth, J.G., Bull. Euro. Physiopath. Resp. (1980) 16:183-195) and reported by Scott et al. (J. Biol. Chem. (1985) 260:7029-7034), both of which are incorporated herein by reference to disclose second order association rate constants.

In general, a value for k_{ass} equal to or greater than 1 x 10^5 $M^{-1}S^{-1}$ for a particular protease-inhibitor reaction is considered to be physiologically significant

(Travis and Salveson Ann. Rev. Biochem. (1983) 52:655-709), incorporated herein by reference to describe the significance of rate constants. Many physiologically important protease-inhibitor reactions such as elastase-α-1-antitrypsin and plasmin-α-2-antiplasmin occur with rate constants as high as $1 \times 10^7\ M^{-1}S^{-1}$ or greater. The thrombin-PN-1 reaction occurs at a similar high rate, or faster, in the presence of heparin.

Native PN-1 has essentially no effect with respect to inhibiting the activity of elastase. However, the R345I variants of the present invention clearly provide not just a new biological activity for the serpin, i.e., its ability to inhibit elastase, but clearly provide an extremely potent elastase inhibitor. The ability of the R345I variants to inhibit elastase to such a degree was in itself a surprising finding. However, it was clearly unexpected to find that, in addition to providing such a potent elastase inhibitor, these variants had still further increased potency with respect to inhibiting elastase while in the presence of heparin.

Variants of the invention are clearly capable not only of providing improved potency with respect to acting as elastase inhibitors, but of providing such activity site-specifically in that their activity is greatly enhanced in the presence of heparin, heparin-like compounds or other related mucopolysaccharides normally found in the endothelial lining of blood vessels. In addition to heparin, a range of sulfated proteoglycans such as other heparin-like compounds normally found on the surface and surrounding extracellular matrix would provide not only increased potency with respect to the ability of the variants of the invention to inhibit elastase but provide site-specific activity due to the affinity of these variants to heparin and heparin-like compounds.

The inhibitory effect of the R345I variant on elastase is increased approximately two orders of magnitude in the presence of heparin. It can be readily determined that "P_1" variants with non-polar residues such as valine substituted for the polar arginine residue could be used as a heparin activatable inhibitor in order to treat individuals suffering from elastase-related diseases such as emphysema, congenital α-1-antitrypsin deficiency, inflammation and septic shock. Non-polar residues which can be used include G, A, V, L, I, M, F and W, and more preferably (due to "R" group structures similar to valine) G, A, V and L, and most preferably I.

In a broad sense, the present invention encompasses PN-1 variants which are capable of acting as potent elastase inhibitors. More specifically, the invention encompasses such PN-1 variants which act as elastase inhibitors and further wherein the ability to inhibit elastase is greatly increased in the presence of heparin and heparin-like materials. Still more specifically, the invention encompasses PN-1 variants which act as elastase inhibitors and which variants have their ability to inhibit elastase increased 10 fold or more in the presence of heparin, preferably 50 fold or more in the presence of heparin and more preferably 100 fold or more in the presence of heparin. Useful formulations of the invention include PN-1 variants formulated in pharmaceutical compositions along with heparin and heparin-like compounds such as various sulfated polysaccharides or proteoglycans. It is particularly preferred if the heparin-like compounds are highly sulfated, thus providing high negative charges.

Above it has been pointed out that P_1 variants of the invention which include non-polar residues such as valine substituted for the polar arginine can be used to treat individuals due to the ability of these P_1 variants

to inhibit the activity of elastase. This is quite surprising since other proteases such as urokinase and plasmin which are inhibited by PN-1 are not heparin activatable. While not wishing to be bound by any particular theory, it may be that the P_1 variants of the present invention are effective in inhibiting elastase due to the cationicity of elastase which promotes its binding to heparin which is anionic. Accordingly, in order to obtain other PN-1 variants which are heparin activatable inhibitors of elastase, the active site should be substituted with other residues which are non-polar and have similar "R" groups in order to have a reasonable expectation of similar activity.

Novel Aspects of PN-1 Variants

The sequence of PN-1α and PN-1β are given in Figures 1 and 2 respectively. Further, factors describing the characteristics of both have been put forth above. Prior to the present disclosure, variants of the invention such as elastase inhibitors of any PN-1 were not known. Further, it was not known whether any such variants would provide any activity, let alone the type of activity obtained. In fact, elastase cleaves native PN-1. This cleavage inactivates PN-1 toward thrombin and other proteases. It was quite possible that, in changing Arg_{345} to Ile(R345I), PN-1 could be changed into an even better substrate for elastase, leading to even quicker inactivation of PN-1. The present invention not only provides variants wherein active sites have been replaced, but shows that such variants have activity and that the activity is substantially different from the activity of the original PN-1. Now that a number of variants and their activity have been shown, it can be seen that still other variants which might possess activity can also be produced. In

connection therewith, it is postulated that variants can be produced wherein substitution is made at both the P_1 and P_1' sites. Such double substitutions could be put forth in a variety of different ways.

One approach to producing such variants is to substitute one of the sites with a residue which is substantially different from the residue present such as including a non-polar residue in place of a polar residue while substituting the other site with a residue which is substantially similar to the residue present there both in terms of being polar or non-polar and in terms of having a similar "R" group. Another approach is to substitute both sites with residues which are substantially different from the original residues. Yet another possible means for producing variants would be to use either of the above-suggested strategies in combination with substituting other sites. A variety of such substitutions will occur to those skilled in the art upon reading this disclosure. What is important is that the resulting variant continued to provide activity. The ability of the variant to provide activity will depend on the substrate specificity. Accordingly, the present invention is intended to encompass single, double and multiple substitutions of the residues to provide variants which continue to have activity with respect to a given protease or gain substantial activity with respect to another protease.

In connection with the present invention, the PN-1 variants which have activity are variants which have (1) substantially increased potency with respect to inhibiting tPA, urokinase, and/or other related enzymes; (2) substantially increased potency with respect to inhibiting elastase; or most preferably (3) substantially increased potency with respect to inhibiting elastase and which potency is still further increased dramatically in

the presence of heparin. In that the present invention has demonstrated that it is possible to produce PN-1 variants which inhibit elastase and has further demonstrated that it is possible to produce such variants which not only inhibit elastase, but have substantially increased potency to inhibit elastase in the presence of heparin others skilled in the art of such inhibitors will be able to deduce other variants which are intended to be within the scope of the present invention.

<u>Specific PN-1 Variants</u>

Examples of protease nexin-1 variants of the invention are listed in Table 2 along with "indication" of the variant.

TABLE 1

Record #	NCY	mutation	sequence	indication
1	NCY2000	CHO PN-1	TTAILIAR--SSPP	
2	NCY2001	P1Ala		
3	NCY2002	P1Arg (WT)		
4	NCY2003	P1Asn		
5	NCY2004	P1Asp		
6	NCY2005	P1Cys		
7	NCY2006	P1Gln		
8	NCY2007	P1Glu		
9	NCY2008	P1Gly		
10	NCY2009	P1His		
11	NCY2010	P1Ile		antielastase
12	NCY2011	P1Leu		antielastase
13	NCY2012	P1Lys		antiplasmin
14	NCY2013	P1Met		
15	NCY2014	P1Phe		
16	NCY2015	P1Pro		
17	NCY2016	P1Ser		
18	NCY2017	P1Thr		
19	NCY2018	P1Trp		
20	NCY2019	P1Tyr		
21	NCY2020	P1Val		antielastase
22	NCY2021	P2Ala (WT)		
23	NCY2022	P2Arg		
24	NCY2023	P2Asn		
25	NCY2024	P2Asp		
26	NCY2025	P2Cys		
27	NCY2026	P2Gln		
28	NCY2027	P2Glu		
29	NCY2028	P2Gly		faster kinetics
30	NCY2029	P2His		
31	NCY2030	P2Ile		
32	NCY2031	P2Leu		
33	NCY2032	P2Lys		
34	NCY2033	P2Met		
35	NCY2034	P2Phe		
36	NCY2035	P2Pro		faster kinetics
37	NCY2036	P2Ser		
38	NCY2037	P2Thr		
39	NCY2038	P2Trp		
40	NCY2039	P2Tyr		
41	NCY2040	P2Val		
42	NCY2041	P3Ala		
43	NCY2042	P3Arg		
44	NCY2043	P3Asn		
45	NCY2044	P3Asp		
46	NCY2045	P3Cys		
47	NCY2046	P3Gln		

Record #	NCY	mutation	sequence	indication
48	NCY2047	P3Glu		
49	NCY2048	P3Gly		
50	NCY2049	P3His		
51	NCY2050	P3Ile (WT)		
52	NCY2051	P3Leu		
53	NCY2052	P3Lys		
54	NCY2053	P3Met		
55	NCY2054	P3Phe		
56	NCY2055	P3Pro		
57	NCY2056	P3Ser		
58	NCY2057	P3Thr		
59	NCY2058	P3Trp		
60	NCY2059	P3Tyr		
61	NCY2060	P3Val		
62	NCY2101	P1'Ala		
63	NCY2102	P1'Arg		
64	NCY2103	P1'Asn		
65	NCY2104	P1'Asp		
66	NCY2105	P1'Cys		
67	NCY2106	P1'Gln		
68	NCY2107	P1'Glu		
69	NCY2108	P1'Gly		
70	NCY2109	P1'His		
71	NCY2110	P1'Ile		anti-Factor Xa
72	NCY2111	P1'Leu		
73	NCY2112	P1'Lys		
74	NCY2113	P1'Met		
75	NCY2114	P1'Phe		
76	NCY2115	P1'Pro		
77	NCY2116	P1'Ser (WT)		
78	NCY2117	P1'Thr		anti-Factor Xa
79	NCY2118	P1'Trp		
80	NCY2119	P1'Tyr		
81	NCY2120	P1'Val		

The above examples 1-81 represent the substitution at different sites within the active site of PN-1. For example, record nos. 2-21 represent a substitution at the P_1 position of PN-1. The indication "WT" is provided to indicate the naturally-occurring or wild-type sequence produce via CHO cells. As is evident from this disclosure, the examples could be continued to include all 20 amino acid substitutions at each position within the active site, that is, all 20 naturally-occurring

amino acids could be substituted, using site-directed mutagenesis, at positions P_1, P_2, P_3, P_4, P_1', P_2', P_3', and P_4'.

It is possible to produce individual type I variants using site-directed mutagenesis. However, it is also possible to produce large numbers of Type I variants at the same time. For example, it is possible to produce the 64 million different variants simultaneously wherein all of the 20 naturally occurring amino acids are substituted at all 8 positions. Such can be carried out using a phage display synthesis methodology as disclosed within U.S. patent 5,223,409 issued June 29, 1993. Further, the chemical synthesis methodology disclosed within U.S. patent 5,010,175 issued April 23, 1991 can be used to produce such large mixtures of variants. Different types of screening methodology such as that disclosed within U.S. patent 5,223,409 can then be used to screen the variants to determine particular activities.

Testing Type I Variants

Any of the variants of the present invention can be tested by comparing the variants with a panel of proteases and determining their second-order rate constants with respect to the different proteases. Such tests have been carried out with an exemplary number of Type I variants, and the results are described below in Tables 1A through 1I.

TABLE 1A
Wild-type (CHO PN-1)
NCY 2000

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	5.98×10^5
2) Plasmin	1.28×10^5
3) Plasmin (hp)	4.51×10^5
4) Xa	7.45×10^3
5) Xa (hp)	4.85×10^4
6) Urokinase	1.49×10^5
7) Urokinase (hp)	3.30×10^5
8) Kallikrein	2.50×10^5
9) Cathepsin G	<100
10) Activated protein C	1.42×10^4
11) Activated protein C (hp)	1.96×10^6
12) Elastase	<100

(hp) indicates in the presence of 10 µg/ml of heparin.

TABLE 1B
(E. coli PN-1)
NCY 2002

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	5.99×10^5
2) Plasmin	3.44×10^5
3) Plasmin (hp)	3.98×10^5
4) Xa	4.82×10^3
5) Xa (hp)	2.48×10^4
6) Urokinase	2.41×10^5
7) Urokinase (hp)	2.35×10^5
8) Kallikrein	6.30×10^4
9) Cathepsin G	<100
10) Activated protein C	8.56×10^2
11) Activated protein C (hp)	2.00×10^5
12) Elastase	<100

(hp) indicates in the presence of 10 µg/ml of heparin.

TABLE 1C
PN-1 (P_2Gly)
NCY 2028

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	5.32×10^4
2) Plasmin	5.52×10^4
3) Plasmin (hp)	6.75×10^4
4) Xa	9.43×10^3
5) Xa (hp)	5.54×10^4
6) Urokinase	1.13×10^5
7) Urokinase (hp)	<100
8) Kallikrein	3.24×10^4
9) Cathepsin G	<100
10) Activated protein C	<100
11) Activated protein C (hp)	1.61×10^5
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 1D
PN-1 (P_2Pro)
NCY 2035

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	1.92×10^4
2) Plasmin	8.48×10^3
3) Plasmin (hp)	1.58×10^4
4) Xa	1.41×10^2
5) Xa (hp)	7.33×10^2
6) Urokinase	<100
7) Urokinase (hp)	<100
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	<100
11) Activated protein C (hp)	1.39×10^5
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 1E
PN-1 (P_1Val)
NCY 2020

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	<100
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	1.20×10^6

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 1F
PN-1 (P_1Ile)
NCY 2010

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	<100
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	4.15×10^6

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 1G
PN-1 (P_1Leu)
NCY 2011

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	<100
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	1.65×10^6

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 1H
PN-1 (P_1Lys)
NCY 2012

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	2.59×10^4
2) Plasmin	1.01×10^5
3) Plasmin (hp)	1.51×10^4
4) Xa	<100
5) Xa (hp)	----
6) Urokinase	1.16×10^4
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	<100
11) Activated protein C (hp)	3.02×10^4
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 11
PN-1 (P_1Met)
NCY 2013

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	----
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	4.81×10^4
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	<100

(hp) indicates in the presence of 10 μg/ml of heparin.

Type II Variants: Serpin Active Site Swap

Type II variants of the invention are produced in a manner similar to Type I variants. However, the active site of PN-1 is modified in a manner so that it matches the sequence of the active site of another protease, and preferably another serpin. Examples of Type II variants of the present invention include the following:

TABLE 2

NCY#	Serpin	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	
	Protease Nexin-1	Leu-	Ile-	Ala-	Arg	Ser-	Ser-	Pro-	Pro	
2203	PAI-1	Val-	Ser-	Ala-	Arg	Met-	Ala-	Pro-	Glu	
2204	PAI-2	Met-	Thr-	Gly-	Arg	Thr-	Gly-	His-	Gly	⊗
2205	PAI-3*	Phe-	Thr-	Phe-	Arg	Ser-	Ala-	Arg-	Leu	
2201	ATIII	Ile-	Ala-	Gly-	Arg	Ser-	Leu-	Asn-	Pro	
2206	α2-anti-plasmin	Ala-	Met-	Ser-	Arg	Met-	Ser-	Leu-	Ser	
2207	C1-inhib-itor	Ser-	Val-	Ala-	Arg	Thr-	Leu-	Leu-	Val	
2208	Kalli-krein BP	Ile-	Leu-	Ser-	Arg	Arg-	Thr-	Ser-	Leu	OR
2209	(rat)	Phe-	Arg-	Ile-	Leu	Ser-	Arg-	Arg-	Thr	
2210	α1AT	Ala-	Ile-	Pro-	Met	Ser-	Ile-	Pro-	Pro	
2211	α1AT related	Glu-	Lys-	Ala-	Trp	Ser-	Lys-	Tyr-	Gln	
2212	α1AC	Leu-	Leu-	Ser-	Ala	Leu-	Val-	Glu-	Thr	OR
		Ile-	Thr-	Leu-	Leu	Ser-	Ala-	Leu-	Val	
2202	HCII	Phe-	Met-	Pro-	Leu	Ser-	Thr-	Glu-	Val	
2213	uro-kinase inh.	Met-	Thr-	Gly-	Arg	Thr-	Gly-	His-	Gly	⊗

⊗ indicates the sequence is preferably followed by -Gly-Pro

Underlining residues indicates a difference from the natural PN-1 sequence.

This table is meant as an example and should not be considered limiting of Type II variants of the invention.

The same methodology referred to above with respect to Type I variants can be used in the production of Type II variants. Further, the methodology disclosed

within the above-cited patents (incorporated herein by reference) can be used to produce Type II variants. The methodology is modified only by first determining the active site of another serpin. After determining the amino acid sequence of the active site of a different serpin and studying the activity of that different serpin it is possible to produce a Type II variant having a particular and desired changed activity as compared with the naturally occurring PN-1.

Testing Type II Variants

As indicated above, all of the variants of the present invention can be tested by determining their second order rate constants relative to a panel of proteases. As with the Type I variants, tests were carried out with a representative number of Type II variants, and the results obtained are put forth below in Tables 2A and 2B.

TABLE 2A
2201 (AT III)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	1.69×10^4
2) Plasmin	2.74×10^4
3) Plasmin (hp)	4.45×10^4
4) Xa	4.21×10^3
5) Xa (hp)	2.98×10^4
6) Urokinase	<100
7) Urokinase (hp)	----
8) Kallikrein	9.49×10^4
9) Cathepsin G	<100
10) Activated protein C	<100
11) Activated protein C (hp)	----
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 2B
2202 (HC II)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	3.36×10^3
2) Plasmin	<100
3) Plasmin (hp)	----
4) Xa	<100
5) Xa (hp)	----
6) Urokinase	<100
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	4.60×10^5
10) Activated protein C	<100
11) Activated protein C (hp)	----
12) Elastase	<100

(hp) indicates in the presence of 10 μg/ml of heparin.

The activity of NCY 2202 (particularly in the presence of heparin) is analogous in some ways to that of NCY 2322 in that both inhibit catheprin G but not elastase. This is completely unexpected due to the similar substrate specificity.

Type III Variants: Incorporation of a Substrate Sequence

Type III variants of the invention are produced by first determining the substrate sequence of a protease. The substrate sequences of some proteases which are known and which would be useful in connection with the present invention are put forth below in Table 3A.

TABLE 3A

PROTEASE	substrate sequence
Thrombin	D-Phe-Pip-Arg-pNA Ts-Gly-Pro-Arg-pNA
Factor Xa	bz-Ile-Glu(γOR)-Gly-Arg-pNA cbo-D-Arg-Gly-Arg-pNA
Factor XIa	Glu-Pro-Arg-pNA
Plasmin	(D/L)-Val-Leu-Lys-pNA D-Val-Phe-Lys-pNA Ts-Gly-Pro-Lys-pNA Glu-Phe-Lys-pNA
Urokinase	Glu-Gly-Arg-pNA Bz-Ala-Gly-Arg-pNA
tPA	(D/L)-Ile-Pro-Arg-pNA
C1-esterase	z-Val-Gly-Arg-pNA
Kallikrein	(D/Bz)-Pro-Phe-Arg-pNA
Neutrophile elastase	Glu-Pro-Val-pNA Ala-Ala-Pro-Val-pNA
Cathepsin G	Ala-Ala-Pro-Phe-pNA Ala-Ala-Pro-Leu-pNA
Pancreatic elastase	Ala-Ala-Ala-pNA

Other substrate sequences can be determined by determining the best artificial small molecule peptide substrates (i.e. Ala-Ala-Pro-Phe-pNA) as determined by k_{cat} and $k\mu$, or by examining the sequence of natural protein substrates (e.g. fibrinogen for thrombin).

After determining the amino acid sequence which a protease will bind to (i.e. its specific substrate sequence), that sequence is used to replace all or a portion of the active site of PN-1. Examples of Type III variants are as follows:

TABLE 3B

Record #	NCY	mutation	sequence	indication
1	NCY2301	XaS	IEGR--	anticoag.
2	NCY2302	Fibrinogen	DPLAGGGGVR--	thrombin inhibition
3	NCY2303	HMWK	SPFR-SVQ	kallikrein inh.
4	NCY2310	FPR	FPR--	thrombin
5	NCY2311	EPV	EPV--	elastase
6	NCY2321	AAPF	AAPF	cathepsin G. inh.
7	NCY2322	AAPL	AAPL--	Elastase
8	NCY2323	AAPV	AAPV--	Elastase
9	NCY2324	AAPI	AAPI--	Elastase

Testing Type III Variants

In Table 3B above, specific Type III variants are shown. Substrate sequences of proteases such as those shown within Table 3A are included within PN-1. These variants were tested against a panel of proteases, and the results are shown below in Tables 3C through 3J.

TABLE 3C
2301 (Xa S)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	<100
2) Plasmin	2.45×10^4
3) Plasmin (hp)	2.54×10^4
4) Xa	1.37×10^3
5) Xa (hp)	6.83×10^3
6) Urokinase	5.71×10^3
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	<100
11) Activated protein C (hp)	----
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 3D
2303 (HMWK)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	2.27×10^3
2) Plasmin	<100
3) Plasmin (hp)	----
4) Xa	<100
5) Xa (hp)	----
6) Urokinase	<100
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	<100
11) Activated protein C (hp)	----
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 3E
2310 (FPR)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	4.13×10^4
2) Plasmin	5.12×10^4
3) Plasmin (hp)	1.32×10^5
4) Xa	3.30×10^2
5) Xa (hp)	----
6) Urokinase	5.30×10^3
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	<100
11) Activated protein C (hp)	4.74×10^5
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 3F
2321 (AAPF)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	7.02×10^{3}
2) Plasmin	<100
3) Plasmin (hp)	----
4) Xa	<100
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	<100

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 3G
2322 (AAPL)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	----
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	4.0×10^{5}
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	<100

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 3H
2324 (AAPI)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	----
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	<100

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 3I
2323 (AAPV)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	----
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	<100

(hp) indicates in the presence of 10 μg/ml of heparin.

TABLE 3J
2311 (EPV)

Protease	Second Order Rate Constant ($M^{-1}s^{-1}$)
1) Thrombin	<100
2) Plasmin	----
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	----
7) Urokinase (hp)	----
8) Kallikrein	<100
9) Cathepsin G	<100
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	<100

(hp) indicates in the presence of 10 μg/ml of heparin.

Type IV Variants

The Type IV variants of the invention are quite different from all of the other variants in that the amino acid sequence may be the same as the naturally-occurring protein or different therefrom. Type IV variants may be generated by covalently binding (i.e., coupling) PEG to any protein, protein fragment, or peptide in general when increased biological stability is desired. Of particular interest are those proteins, protein fragments, and peptides which are useful in therapeutic applications, such as serine protease inhibitor proteins, growth factors, and cytokines. The proteins PN-1, human growth hormone (hGH), erythropoietin (EPO), and antithrombin-III (ATIII) are of particular interest. Specific exemplary proteins of interest, as well as exemplary classes of proteins, which can be modified to create a Type IV variant using the methods of the invention are provided in Table 4A.

TABLE 4A

Exemplary Proteins and Protein Classes for Generation of Type IV Variants

SPECIFIC EXEMPLARY PROTEINS	
interferon-α2A	insulin-like growth factor-1 (IGF-1)
interferon-α2B	insulin
human growth hormone (hGH)	transforming growth factor (TGF)
erythropoietin (EPO)	ciliary neurite transforming factor (CNTF)
thrombopoietin (TPO)	brain-derived neurite factor (BDNF)
IL-1	insulintropin
IL-2	glial-derived neurite factor (GDNF)*
IL-1 RA	tissue plasminogen activator (tPA)
superoxide dismutase (SOD)	urokinase
catalase	streptokinase
fibroblast growth factor (FGF) (acidic or basic)	hemoglobin
neurite growth factor (NGF)	adenosine deamidase
granulocyte macrophage colony stimulating factor (GM-CSF)	bovine growth hormone (BGH)
granulocyte colony stimulating factor (G-CSF)	calcitonin
platelet derived growth factor (PDGF)	bactericidal/permeability increasing protein (BPI)
L-asparaginase	arginase
uricase	phenylalanine
γ-interferon	ammonia lyase

TABLE 4A (cont.)

EXEMPLARY CLASSES OF PROTEINS	
proteases	pituitary hormones
protease inhibitors	growth factors
cytokines	somatomedians
chemokines	immunoglobulins
gonadotrophins	interleukins
chemotactins	interferons
lipid-binding proteins	allergens

* GDNF is the same protein as protease nexin-1 (PN-1).

Type IV variants are created by attaching polyethylene glycol to a thio group on a cysteine residue of the protein. The PEG moiety attached to the protein may range in molecular weight from about 200 to 20,000 MW. Preferably the PEG moiety will be from about 1,000 to 8,000 MW, more preferably from about 3,250 to 5,000, most preferably 5,000 MW.

General methods of attaching polyethylene glycol to proteins are disclosed within U.S. Patent 4,179,337 issued December 18, 1979 (incorporated herein by reference to disclose methods of attaching polyethylene glycol to proteins). Further, other methods of attaching polyethylene glycol are disclosed within U.S. Patent 5,122,614 issued June 16, 1992, also incorporated herein by reference to disclose methods of attaching polyethylene glycol to proteins.

The present inventors have discovered that novel modified proteins can be created by attaching the polyethylene glycol to a cysteine residue within the protein. Preferably, the protein is modified by attaching the polyethylene glycol to a cysteine residue at a position which is normally glycosylated. In another preferred embodiment, a cysteine residue is added at a

position which is normally glycosylated (e.g. N-glycosylated), and the polyethylene glycol is attached to the thio group of the added cysteine residue. In addition, if the protein of interest is one member of a family of structurally related proteins, glycosylation sites for any other member can be matched to an amino acid on the protein of interest, and that amino acid changed to cysteine for attachment of the polyethylene glycol. Alternatively, if a crystal structure has been determined for the protein of interest or a related protein, surface residues away from the active site or binding site can be changed to cysteine for the attachment of polyethylene glycol.

It has also been found that it is possible to attach other groups to the thio group of the cysteine residue. For example, the protein may be biotinylated by attaching biotin to a thio group of a cysteine residue. Examples of Type IV variants are as follows:

TABLE 4

Record #	NCY	mutation	sequence	indication
1	NCY2601	PEG-PN1	lys-modified	long half-life
2	NCY2611	Biotin-PN1	lys-modified	detection/ coupling
3	NCY2621	PEG-PN1	cys-modified	long half-life
4	NCY2631	Biotin-PN1	cys-modified	detection/ coupling

Of the above examples, records numbers 1 and 2 are of a general type known in the art in that the polyethylene glycol or biotin is attached to lysine position of the peptide. However, record numbers 3 and 4 are, respectively, examples wherein the polyethylene glycol or biotin are connected at a cysteine group. The importance of such is described further below.

The addition of polyethylene glycol (PEG) to proteins is a common method to increase serum half-life and decrease immunogenicity and antigenicity. Nucci et al. describe several proteins which have been modified by addition of PEG including adenosine deamidase, L-asparaginase for treatment of acute lymphoblastic leukemia, interferon alpha 2b (IFN-α2b) as an anticancer and antiviral drug, superoxide dismutase as an anticancer drug, streptokinase, tPA, urokinase, uricase, hemoglobin, interleukins, interferons, TGF-β, EGF, and other growth factors (Nucci et al., 1991, *Adv. Drug Delivery Rev.* 6:133-151).

Typically, this modification of a protein by attachment of a PEG moiety involves activating PEG with a functional group which will react with lysine residues on the surface of the protein. If the modification of the protein goes to completion, the activity of the protein is usually lost. Current modification procedures allow only partial PEGylation of the protein. Usually this results in only 50% loss of activity and greatly increased serum half-life, so that the overall dose of the protein required for the desired activity is lower.

An unavoidable result of the partial modification is that there will be a statistical distribution of the number of PEG groups per protein, each PEG attached to a lysine residue. Also, there will be a random usage of lysine residues on the surface. For instance, when adenosine deaminase is optimally modified, there is a loss of 50% activity when the protein has about 14 PEG per protein, with a broad distribution of actual PEG per individual protein and a broad distribution of actual lysine residues used.

Early work on PEG modification relied on activating PEG with cyanuric chloride and the coupling with proteins. This approach suffers from several

disadvantages, most noteworthy are the toxicity of cyanuric chloride and loss of activity. There have been significant advances recently to reduce the toxicity of the chemicals involved in the PEGylation reactions. Zalipsky, et al. (Zalipsky, S., Seltezer, R. & Nho, K. (1991) Polymeric Drugs and Drug Delivery Systems. ACS.) describe the use of succinimydyl carbonates of PEG to produce stable carbamate linkages with proteins via free lysine residues. There are several other methods for modification of proteins with PEG through free lysine residues, but each suffers from the problems associated with partial, random modification of proteins and the potential for losing activity if lysine residues are important.

The current state of the art of PEGylation technology could be greatly improved upon if the reaction could be made more specific. For instance, if the reactive region of the protein could be blocked during PEGylation, it is expected that the protein would retain all activity even after saturation modification. This may prove to be expensive and inconvenient (except if the modification took place during an affinity purification step where the active site is protected, and the protein is purified all in one step). In some cases to block the active site region would lead to irrecoverable loss of activity, and so this method could not be employed.

Another alternative is to PEG-modify other residues such as His, Trp, Cys, Asp, Glu, etc. in such a manner that activity is not lost. It is anticipated or likely that many of these residues will be at or near the active site or that these residues will not be at the surface or in sufficient number to significantly affect serum half-life, or that the modification chemistry is not specific enough for the target residue, or the

chemistry is too harsh for the protein to withstand without loss of activity.

The most desirable situation would be if there were a surface distribution of an amino acid which is reactive and can be modified easily and specifically and is away from the active site region. Cysteine is an ideal candidate for such modification since it is rarely used at or near the active site (except cysteine proteases) and modification chemistry has been well established. Maleimido-PEG is perhaps the most useful, but other chemistries are available for specific cysteine modification. Using site-directed mutagenesis, new cysteine residues can be added on the surface, away from the active site region. This is most conveniently and easily done if a X-ray crystal structure is known.

Even with this protein engineering strategy it is not easily known a *priori* which surface residues to change to cysteine and how many PEG modification sites to add in order to increase stability sufficiently. There are many strategies, among them to create 20 or more independent single-site cysteine mutations, PEGylate each independently and analyze each for remaining activity. Mutant proteins which retain sufficient activity can be combined to generate one protein with multiple PEG attachment sites.

There is another strategy to identify good PEG attachment sites which has the advantage that one does not require knowledge of the three dimensional structure, and also takes advantage of the selective power of evolution to weed out bad sites. Nature has chosen to add glycosylation residues to the surface of secreted proteins to aid stability and increase serum half-life. For example, asparagine residues are glycosylated when part of a well known N-glycosylation signal (NP!T/SP!). Replacement of these Asn residues by cysteine, followed

by cysteine-specific PEGylation is expected to lead to proteins with significantly increased serum half-life with a minimum loss in activity. To date, this is the closest thing to _in vitro_ glycosylation, and in some respects may be better since glycosylation with inappropriate sugar residues may lead to _increased_ clearance from the serum by the liver, whereas PEG residues are expected to be rather inert.

If a higher degree of PEG modification is desired and the protein to be modified is one member of a family of structurally related proteins, other members of the same family will often have one or more sites of glycosylation not found in the protein of interest. If these "new" potential sites are in a region which is reasonably conserved (i.e. not part of an insertion or with a sequence which is so different that it is likely to have a different structure) it is expected that replacement of the residue equivalent to the Asn with cysteine followed by PEGylation will result in a more highly PEG modified protein without significant loss in activity.

If a further higher degree of PEG modification is required, other solvent accessible residues can be changed to cysteine, and the resultant protein subjected to PEGylation. Appropriate residues can easily be determined by those skilled in the art. For instance, if a three-dimensional structure is available for the protein of interest, or a related protein, solvent accessible amino acids are easily identified. Also, charged amino acids such as Lys, Arg, Asp and Glu are almost exclusively found on the surface of proteins. Substitution of one, two or many of these residues with cysteine will provide additional sites for PEG attachment. In addition, amino acid sequences in the native protein which are recognized by antibodies are

usually on the surface of the protein. These and other methods for determining solvent accessible amino acids are well known to those skilled in the art.

Modification of proteins with PEG can also be used to generate dimers and multimeric complexes of proteins, fragments, and/or peptides which have increased biological stability potency. These dimeric and multimeric proteins of the invention may be naturally occurring dimeric or multimeric proteins. For example, the dimer or multimer may be composed of cross-linked subunits of a protein (e.g., hemoglobin). Alternatively, the dimeric and multimeric proteins may be composed of two proteins which are not normally cross-linked (e.g., a dimer of cross-linked EPO protein.

Dimeric proteins of the invention may be produced by reacting the protein with $(Maleimido)_2$-PEG, a reagent composed of PEG having two protein-reactive moieties. This PEGylation reaction with the bi-functional PEG moiety generates dimers of the general formula:

$$R_1\text{-S-PEG-S-}R_2$$

where R_1 and R_2 may represent the same or different proteins and S represents the thio group of a cysteine either present in the native R_1 or R_2 protein, or introduced by site-directed mutagenesis. The proteins R_1 and R_2 may each vary in size from about 6 to 1,000 amino acids, preferably about 20 to 400 amino acids, more preferably 40 to 200 amino acids. In dimeric molecules, R_1 and R_2 are preferably from about 100 to 200 amino acids. Dimers and multimers of particular interest include those composed of proteins, protein fragments and/or peptides which are less than about 40,000 molecular weight.

Where the protein contains (or is engineered to contain) more than one free cysteine, multimeric proteins

where the proteins (represented by R_1, R_2,.....R_n) may be the same or different can be generated. The proteins represented by R_1, R_2,.....R_n may vary in size from about 6 to 1,000 amino acids, preferably 20 to 400 amino acids, more preferably 40 to 200 amino acids. Such multimeric proteins may be of the following general formula:

$$R_1\text{-}(\text{-S-PEG-S-}R_2)_n$$

where R_1 represents the protein having multiple free cysteines, for example from 2 to 20 free cysteines, usually from 5 to 7 free cysteines. R_2 may represent a protein the same as or different from R_1. Furthermore, each of the R_2 proteins attached to R_1 may be the same proteins, or represent several different proteins.

The degree of multimeric cross-linking can be controlled by the number of cysteines either present and/or engineering into the protein and by the concentration of (Maleimido)$_2$PEG used in the reaction mixture. In addition, the Maleimido-PEG+ (Maleimido)$_2$-PEG reagents may be used in the same reaction with proteins for formation of couplings of proteins having simple PEG moieties as well as PEG cross-links between proteins within the complex. The dimeric or multimeric protein generated will have an increased half-life relative to the native, protein, due at least in part to its increased size relative to the native protein. Such larger proteins are not degraded or cleared from the circulation by the kidneys as quickly as are smaller proteins. In addition, activity or potency of the dimeric or multimeric protein may be increased.

Dimeric and multimeric proteins may be generated by reaction with Maleimido-PEG or (Maleimido)$_2$-PEG. Exemplary proteins for dimeric and/or multimeric complex formation using the method of the invention include PN-1, PN-1 variants, hemoglobin, and erythropoietin (EPO), as well as any of the proteins or members of the protein

classes exemplified in Table 4A. Preferably, the protein generated by the method of the invention is a PEGylated cross-linked complex of the "a" and "b" chains of hemoglobin, multimeric complexes of hemoglobin having intermolecular and/or intramolecular cross-links may also be generated by the subject method.

In general, the method of identifying cysteine residues for PEG modification, and/or identifying amino acid residues to be replaced with cysteine which are subsequently modified by attachment of PEG, provides for generation of a PEGylated protein which can be reasonably expected to retain most or all of the activity of the native protein. The sites selected for modification and/or substitution with cysteine are selected on the basis of the structure of the protein, i.e. the selected sites are solvent accessible residues which are not involved in the active site.

The effect of mutations located outside of the active site are generally predictable in that they generally do not change the primary activity of the protein. In addition, the structural mutations described herein are within solvent-accessible regions of the protein (i.e. on the protein "surface") which have limited or no interaction with other residues in the protein molecule. Thus, mutations at these positions are unlikely to affect the conformation of any other amino acid in a protein.

<u>Type V Variants</u>

Type V variants of the invention are produced by fusing all or a fragment of another protein to PN-1. Preferably, the amino terminal fragment of a protein such as urokinase is fused to PN-1 in order to localize PN-1 to a different receptor, i.e. to the urokinase receptor. In addition to using urokinase, it is possible to fuse

the amino terminal fragment of proteins such as tPA, Factor IX, Factor X, and Protein C. Examples of Type V variants are as follows:

TABLE 5

Record #	NCY	mutation	sequence	indication
1	NCY2501	ATF-PN1	urokinaseATF	anti-metastasis
2	NCY2502	HSA-PN1	HSA chimera	long half-life
3	NCY2503	IgG-PN1	IgG chimera	long half-life
4	NCY2504	F9-PN1	Factor IX chimera	anti-coagulation
5	NCY2505	F10-PN1	Factor X chimera	anti-coagulation
6	NCY2506	APC-PN1	Protein C chimera	pro-coagulant

The type V variants of the invention can be produced in manner similar to variants of type I, II and III. However, it is more preferable to produce such variants by chemically fusing an N-terminal fragment of a different protein to the PN-1.

The Type V variants can also be produced by chemical linkage of purified preparations of both protein components. Such linkage is conveniently accomplished by using bi-functional cross-linking reagents. Methods for chemically establishing such linkages are well known to those skilled in the art. Specific variants which might be produced in this manner include variants produced by fusing PN-1 to any one of: EGF; Factor IX; Factor X; and APC.

The method of PEGylation of the invention is intended to be a general procedure and as such is applicable to any protein to increase solubility, circulating half life and/or to decrease immunogencity.

Testing Type V Variants

The Type V variants of the invention are chimeric proteins wherein PN-1 is covalently bound to another protein. The same panel of proteases indicated above were used to test one of the Type V variants of the invention, and the results are put forth below.

TABLE 5A

2501 (ATF-PN-1)

Protease	Second Order Rate Constant ($M^{-1}S^{-1}$)
1) Thrombin	5.8×10^5
2) Plasmin	3.5×10^5
3) Plasmin (hp)	----
4) Xa	----
5) Xa (hp)	----
6) Urokinase	2.3×10^5
7) Urokinase (hp)	----
8) Kallikrein	----
9) Cathepsin G	----
10) Activated protein C	----
11) Activated protein C (hp)	----
12) Elastase	----

(hp) indicates in the presence of 10 μg/ml of heparin.

Use and Administration

The different chimeric proteins, PN-1 variants, and cysteine-PEGylated proteins of the invention (as indicated above) can provide different effects. For example, P_1 variants with non-polar residues such as valine substituted for the polar arginine residue could be used as heparin activatable inhibitors. Such inhibitors could be used to treat individuals suffering from elastase-related diseases. Although not limited to such diseases, such variants could be used to treat

emphysema, congenital α-1-antitrypsin deficiency, inflammation, arthritis and septic shock.

One of the most important and immediate perceived uses of the chimeric proteins, PN-1 variants, and/or cysteine-PEGylated versions of these proteins of the invention is the inclusion of such within various topical formulations such as creams or gels, or a combination of such formulations, with various bandages for application to wounds to aid in wound healing and decrease inflammation at wound sites. In that chimeric proteins, PN-1 variants, and cysteine-PEGylated versions of these proteins of the invention are believed to be effective in decreasing inflammation, injectable formulations containing the chimeric proteins, PN-1 variants, and/or cysteine-PEGylated versions of these proteins of the invention may be injected directly into inflamed joints or other inflamed areas of the body in order to decrease the inflammation. Further the formulations of the invention may be used prophylactically by providing the chimeric proteins, PN-1 variants, and/or cysteine-PEGylated versions of these proteins to a particular site which may be subjected to trauma, (such as in surgery), and thus inflammation, to prevent the inflammation from occurring.

Generally, the pharmaceutical compositions containing the chimeric proteins, PN-1 variants, and/or cysteine-PEGylated proteins of the invention will be formulated in a non-toxic, inert, pharmaceutically acceptable aqueous carrier medium, preferably at a pH of about 5 to 8, more preferably 6 to 8, although the preferred pH of the pharmaceutical composition may vary according to the protein employed and condition to be treated.

Of particular interest in the present invention are cysteine-PEGylated proteins and pharmaceutical

compositions containing these protein. PEGylation of proteins generates proteins which are ready for immediate therapeutic use (i.e. do not require reconstitution), have increased solubility and have an increased half-life and are reduced in immunogenicity and antigenicity relative to the unmodified protein (Nucci et al. 1991 *Adv. Drug Delivery res.* **6**:133-151). The increased half-life of PEGylated proteins decreases the amount of protein needed for an effective dosage, reduces the number and frequency of administrations required, and decreases the patient's exposure to the protein, thus decreasing the potential for allergic reactions, toxic effects, or other side effect. These characteristics of PEGylated proteins allow for long-term use of the protein with less potential for undesirable side effects related to protein immunogenicity and/or toxicity. Exemplary proteins for which an increase half-life has effected by PEGylation of the protein include: hGH, insulin, interferon-alpha2A (IFN-alpha-2A), interferon-alpha2B (IFN-alpha-2B), tPA, EPO, G-CSF, antigen E, arginase, asparaginase, adenosine deaminase, batroxobin, bovine serum albumin, catalase, elastase, factor VIII, galactosidase, alpha-galactosidase, beta-glucuronidase, IgG, honeybee venom, hemoglobin, interleukin-2, lipase, phenylalanine ammonia lyase, $alpha_1$-proteinase inhibitor, pro-urokinase, purine nucleoside phosphorylase, ragweed allergen, streptokinase, superoxide dismutase, tPA, D-alpha-tocopherol, trypsin, tryptophanase, uricase, and urokinase (see in general Nucci et al. *ibid.*; see also Davis et al. 1981 *Clin. Exp. Immunol.* **46**:649-652 (bovine adenosine deaminase); Nishimura, et al. 1985 *Life Sci.* **33**:1467-1473 (batroxobin); Savoca et al. 1979 *Biochimica et Biophysica ACTA* **578**:47-53 (arginase); Till, et al. 1983 *J. Trauma* **23**:269-277 (asparaginase); Veronese, et al. 1983 *J. Pharm. Pharmacol.* **35**:281-283 (superoxide

dismutase); Davis et al. 1981 *Lancet* 2:281-283 (urate oxidase); and Dellinger et al. 1976 *Cancer* 38:1843-1846).

PEGylated proteins may be administered for the treatment of a wide variety of diseases. Exemplary disease conditions and the proteins useful in treatment of these diseases are provided in Table 6A.

TABLE 6A
Exemplary Disease Conditions Amenable to Protein Therapy

Enzyme Deficiency adenosine deaminase[1] Purine nucleotide phosphorylase Galactosidase β-glucuronidase	**Endotoxic Shock/Sepsis** Bactericidal/permeability increasing protein Lipid-binding protein (LBP)
Antioxidants for Cancer Therapy Superoxide dismutae Catalase	**Blood Protein Replacement Therapy** Hemoglobin Albumin
Cancer Interferon-α Interferon-γ IL1-α Phenylalanine ammonia lyase Arginase L-asparaginase Uricase Granulocyte colony stimulating factor (GCSF) Monoclonal antibodies Tissue necrosis factor	**Growth Factors (for use in wound healing, induction of red blood cell formation, etc.)** Epidermal growth factor G-CSF Interferon-γ Transforming growth factor EPO Thrompoietin Insulin-like growth factor-1 Insulin hGH
Cardiovascular Disease Tissue Plasminogen Activator Streptokinase (native or chimeric) Urokinase (native or chimeric) α-antitrypsin antithrombin-III Other proteases or protease inhibitors Apolipoptoreins (particularly B-48) Circulating Scavenger Receptor APO A1[2]	
[1] For treatment of severe combined immunodeficiency	
[2] Converts low-density lipoproteins to high-density lipoproteins	

As discussed above, chimeric proteins, PN-1 variants, and cysteine-PEGylated proteins may be delivered within the formulations and by the routes of administration discussed above. The particular formulation, exact dosage, and route of administration will be determined by the attending physician and will vary according to each specific situation. Such determinations are made by considering such variables as the condition to be treated, the protein to be administered, the pharmacokinetic profile of the particular protein, as well a various factors which may modify the effectiveness of the protein as a drug, such as disease state (e.g. severity) of the patient, age, weight, gender, diet, time of administration, drug combination, reaction sensitivities, tolerance to therapy, and response to therapy. Long-acting protein drugs might only be administered every 3 to 4 days, every week or once every two weeks. Where cysteine-PEGylated proteins are used in the pharmaceutical composition, the clearance rate (i.e. the half-life of the protein) can be varied to give ultimate flexibility to fit the particular need of the patient by changing, for example, the number of PEG moieties on the cysteine-protein, the size of the PEG moiety

Where cysteine-PEGylated proteins are employed, the daily regimen should generally be in the range of the dosage for the natural, recombinant, or PEGylated protein. Normal dosage amounts may vary from 0.1 to 100 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages for particular proteins is provided in the literature with respect to the administration of either native proteins and/or proteins PEGylated by conventional methodologies. For example, guidance for administration of antithrombin-III for the prevention of

fibrin clot formation may be found in USPNs 5,292,724 and 5,182,259; guidance for administration of human growth hormone (hGH) in the treatment of individuals intoxicated with poisonous substances may be found in USPNs 5,140,008 and 4,816,439; guidance for administration of hGH in the treatment of topical ulcers may be found in USPN 5,006,509; guidance for administration of (EPO) for treatment of anemia and pulmonary administration of EPO may be found in USPN 5,354,934; guidance for administration of EPO, GM-CSF, G-CSF, and multi-CSF for treatment of pancytopenia may be found in USPN 5,198,417; guidance for administration of EPO for treating iron overload may be found in USPN 5,013,718; guidance for administration of EPO in the treatment of hemoglobinopathies may be found in USPN 4,965,251; guidance for administration of insulin the treatment of diabetes may be found in USPN 4,478,822; guidance for delivery of asparaginase for treatment of neoplasms may be found in USPNs 4,478,822 and 4,474,752; guidance for administration of L-asparaginase in the treatment of tumors is found in USPN 5,290,773; guidance for administration of prostaglandin E1, prostaglandin E2, prostaglandin F2 alpha, prostaglandin I2, pepsin, pancreatin, rennin, papain, trypsin, pancrelipase, chymopapain, bromelain, chymotrypsin, streptokinase, urokinase, tissue plasminogen activator, fibrinolysin, deoxyribonuclease, sutilains, collagenase, asparaginase, or heparin in a cryogel bandage for treatment of sites of trauma may be found in USPN 5,260,066; guidance for the administration of superoxide dismutase, glucocerebrosidase, asparaginase, adenosine deaminase, interferons (alpha, beta, and gamma), interleukin (1,2,3,4,5,6,7), tissue necrosis factor (TNF-alpha or TNF-beta), and colony stimulating factors (CSF, G-CSF, GM-CSF) in liposomes may be found in USPN 5,225,212;

guidance for administration of asparaginase or insulin in the treatment of neoplastic lesions may be found in USPN 4,978,332; guidance for administration of asparaginase in the reduction of tumor growth may be found in USPN 4,863,910; guidance for the administration of antibodies in the prevention of transplant rejection may be found in USPNs 4,657,760 and 5,654,210; guidance for the administration of interleukin-1 as a therapy for immunomodulatory conditions including T cell mutagenesis, induction of cytotoxic T cells, augmentation of natural killer cell activity, induction of interferon-gamma, restoration or enhancement of cellular immunity, and augmentation of cell-mediated anti-tumor activity may be found in USPN 5,206,344; guidance for the administration of interleukin-2 in the treatment of tumors may be found in USPN 4,690,915; and guidance for administration of interleukin-3 in the stimulation of hematopoiesis, as a cancer chemotherapy, and in the treatment of immune disorders may be found in USPN 5,166,322.

All U.S. patents cited hereinabove are incorporated herein by reference with respect to the guidance provided in administration of the particular protein and/or PEGylated protein described therein.

Several PEGylated proteins have already been approved for use by the U.S. Food and Drug Administration (FDA). These PEGylated proteins include: hGH, insulin, interferon-alpha2A, interferon-alpha2B, tPA, EPO, G-CSF, and a hepatitis B vaccine which contains PEGylated proteins (Nucci et al. *ibid*).

Due to the usefulness of PEGylated proteins in therapy, there is a clear need for a method of generating PEGylated proteins at specific sites and which allows for the precise selection of amino acid residues for PEGylation, thus increasing the likelihood of generation

of PEGylated proteins which retain the activity of the unmodified parent protein.

It is pointed out that PN-1 is not found in significant quantities in plasma and may function primarily in tissues. The high affinity heparin binding site of PN-1 appears to serve to localize PN-1 to connective tissues and cells which contain sulfated proteoglycans on their surface and surrounding extracellular matrix. Thus, the primary role of PN-1 seems to be in regulating proteolytic activity in tissues as opposed to blood. In that PN-1 is found in brain tissue another aspect of the invention involves delivering formulations of the invention containing PN-1 variants or chimeric proteins in order to facilitate peripheral or central nerve regeneration. Formulations, routes of administration and dosages for use of PN-1 in the treatment of inflammation and wounds are described in USPNs 5,206,017; 5,196,196; and 5,112,608; each of which are incorporated herein by reference to the extent that such methods of treatment using PN-1 are described.

It is generally not possible to obtain desirable results by administering large protein compounds such as chimeric proteins or protease nexin-1 and its variants by oral delivery systems. Such proteins are generally digested in the GI tract (unless formulated with special carriers) and do not enter the cardiovascular system in their original forms due to such digestion. Chimeric proteins, PN-1 variants, and/or cysteine-PEGylated versions of these proteins can be administered by any type of injection, such as intramuscular or intravenous, thus avoiding the GI tract. Other modes of administration include transdermal and transmucosal administrations provided by patches and/or topical cream compositions. Transmucosal administrations can include nasal spray formulations which include the chimeric

proteins, protease nexin-1 variant, and/or cysteine-PEGylated proteins within a nasal formulation which contacts the nasal membranes and diffuses through those membranes directly into the cardiovascular system. PEGylated proteins may have an increased ability to cross membranes and thus may enter the body more easily. Formulations which include the chimeric proteins, PN-1 variants within aerosols for intrapulmonary delivery are also contemplated by this invention, as are intraocular delivery systems wherein the chimeric proteins or PN-1 variants are included within ophthalmic formulations for delivery in the form of eye drops.

Any of the above suggested means of administration could be provided in a variety of different formulations. The formulations can be designed to provide the chimeric proteins or PN-1 variants systemically or to a particular site. Further, the formulations can be designed so as to provide the chimeric proteins or PN-1 variants as quickly as possible or in a sustained release or timed released manner. For example, topical formulations could be created whereby the chimeric proteins or PN-1 variants of the invention were incorporated or disbursed throughout topical polymer formulations capable of slowly releasing the chimeric proteins or PN-1 variants to a wound site in order to continually aid in wound healing and in preventing inflammation.

As indicated above, different formulations of the invention can be administered in a variety of different manners in order to introduce the chimeric proteins, PN-1 variants, and/or cysteine-PEGylated protein into the cardio vascular system. The chimeric proteins, PN-1 variants, and/or cysteine-PEGylated proteins are administered for a variety of purposes which generally relate to, for example: blocking proteolytic activity; inhibition of tumor growth or metastasis; promotion of

wound healing and/or nerve fiber regeneration; replacement therapy for protein-deficient states (e.g. diabetes); inhibition of bacterial, fungal, or viral growth; enhancement of the immune response; induction of maturation of bone marrow stem cells (e.g. in bone marrow transfers); regulation of blood clotting; treatment of inflammation; or treatment of bacterial sepsis and endotoxic shock; replacement of albumin or hemoglobin (e.g. to replace blood transfusions). In particular, intravenous formulations containing the chimeric proteins, PN-1 variants, and/or cysteine-PEGylated versions of these proteins are useful for their anti-thrombolytic effect and therefore can be administered to aid and a prevention and/or alleviation of strokes and/or heart attacks.

EXAMPLES

The following examples are provided so as to give those of ordinary skill in the art a complete disclosure and description of how to make and use the PN-1 variants of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to insure accuracy with respect to the specifics given such as the association rate constants and temperature but some experimental errors and deviations should be accounted for. With respect to the formulation examples, parts are parts by weight, and any temperature readings are in degrees centigrade and all experiments were carried out at or near atmospheric pressure.

EXAMPLE A

The Synthesis of PN-1

PN-1 was purified to homogeneity from serum-free medium conditioned by human foreskin fibroblasts in microcarrier cultures by affinity chromatography on heparin-agarose, followed by gel exclusion chromatography, as described in detail by Scott, R.W. et al., J Biol Chem (1985) 260:7029-7034, incorporated herein by reference. Of course, other chromatographic supports which contain heparin for affinity binding or other matrix such as cm sepharose or S-sepharose can also be used. The purified protein shows an M_r of 42-43 kd, based on sedimentation equilibrium analysis, or of 47 kd, estimated from gel-exclusion chromatography. The purified material shows the properties exhibited by PN-1 when contained in conditioned medium, including formation of sodium dodecylsulfate-stable complexes with thrombin, urokinase, and plasmin; inhibition of protease activity; heparin-enhanced inhibition of thrombin; and cellular binding of protease-PN complexes in a heparin-sensitive reaction. The N-terminal amino acid sequence of the isolated, purified protease nexin was determined for the first 34 amino acids to be: Ser-His-Phe-Asn-Pro-Leu-Ser-Leu-Glu-Glu-Leu-Gly-Ser-Asn-Thr-Gly-Ile-Gln-Val-Phe-Asn-Gln-Ile-Val-Lys-Ser-Arg-Pro-His-Asp-Asn-Ile-Val-Ile.

The PN-1 variants of the present invention can be synthesized by utilizing the pure PN-1 which has been isolated and purified in the manner indicated above. The variants can be obtained by cleaving the purified PN-1 protein at the P_1 or P_1' site and replacing the arginine, serine or both residues at that site with the desired non-polar substitute residue. After replacement of the desired residue with the desired non-polar residue, the segments can be fused utilizing protocols known to those skilled in the art. Although such methodology could be

utilized in order to obtain the variants of the present invention, this methodology is somewhat cumbersome and is extremely limited, due to the very small amounts of PN-1 which can be extracted and purified. Accordingly, although the above procedure could be utilized, it is not the preferred method of making PN-1 or the variants disclosed herein. PN-1 and its variants are generally produced utilizing recombinant technology, as described below.

EXAMPLE B

A Generalized Recombinant Synthesis of PN-1

Methods of producing protease nexin-1 utilizing recombinant technology are disclosed within published European patent application 873049126 which published application is incorporated herein by reference to disclose recombinant technologies utilized in producing protease nexin-1. The procedure can be modified by those skilled in the art, reading this disclosure, to obtain PN-1 variants.

cDNA encoding the complete human PN-1 protein was obtained from a foreskin fibroblast DNA library. The retrieval of this clone took advantage of probes based on the amino acid sequence determined in the native protein. The cloned cDNA is amenable to expression in recombinant cells of both procaryotic and eucaryotic organisms by excising the coding sequence from the carrier vector and ligating it into suitable expression systems.

The PN-1 can be directly produced as a mature protein preceded by a Met N-terminal amino acid (which may or may not be processed, depending on the choice of expression systems) may be produced as a fusion protein to any desirable additional N-terminal or C-terminal sequence, or may be secreted as a mature protein when preceded by a signal sequence, either its own, or a

heterologous sequence provided by, for example, the known signal sequence associated with the bacterial-lactamase gene or with secreted human genes such as insulin or growth hormones. Means for providing suitable restriction sites at appropriate locations with respect to the desired coding sequence by site-directed mutagenesis are well understood, and the coding sequence can thus be provided with suitable sites for attachment to signal sequence or fusion sequence, or into expression vectors.

If bacterial hosts are chosen, it is likely that the protein will be produced in nonglycosylated form. If the PN-1 is produced intracellularly as a "mature" protein, the N-terminal methionine may be only partially processed, or not processed at all. Thus, the protein produced may include the N-terminal Met. Modification of the protein produced either intracellularly or as secreted from such bacterial host can be done by providing the polysaccharide substances, by refolding using techniques to sever and reform disulfide bonds, or other post-translational ex vivo processing techniques. If the protein is produced in mammalian or other eucaryotic hosts, the cellular environment is such that post-translational processing can occur in vivo, and a glycosylated form of the protein is most likely produced.

The recombinant cells are cultured under conditions suitable for the host in question, and the protein is recovered from the cellular lysate or from the medium, as determined by mode of expression. Purification of the protein can be achieved using methods similar to that disclosed by Scott, R.W. et al., J Biol Chem (supra), or by other means known in the art.

Once DNA segments coding for the production of PN-1 have been inserted into bacterial hosts, multiple copies of the segments can, of course, be cloned by

growing the bacteria. The segments can be extracted from the bacteria by the use of conventional methodology whereby the DNA is extracted by subjecting disrupted cells to centrifugation and then subjecting the extracted DNA to enzyme digestion, which will result in obtaining the desired segments by subjecting the digested DNA to separation processes such as gel electrophoresis and blotting. The segments coding for the production of PN-1 can then be subjected to conventional recombinant methodologies in order to substitute codons coding for the arginine and/or serine with new codons which code for the production of the desired non-polar amino acid residue. Once such recombinant segments are produced, they can be reinserted into vectors and hosts in the manner described above in order to obtain the production of the desired PN-1 variants. A variety of vector and host systems known to those skilled in the art can be used.

In addition, it is pointed out that PN-1 variants might be made by using recombinantly produced PN-1 and then substituting only the desired "R" group (e.g., -OH of serine 346) with a non-polar "R" group (e.g., $-CH_2CH_2-s-CH_3$) to get a PN-Met_{346} variant. Such replacements of the "R" group can be carried out using published protocols known to those skilled in the art.

EXAMPLE C

Production of Recombinant PN-1 Variants in Insect Cells Using a Baculovirus Expression System

C.1. Construction of plasmid expression vector:

In order to produce PN-1 and/or PN-1 variants in insect cells, the cDNA sequence must first be inserted into a suitable plasmid expression vector, such as pAC373. Appropriate restriction sites for this insertion

can be created by standard site-directed mutagenesis procedures. The essential properties of a suitable expression vector include a transcriptional promoter such as the polyhedron gene promoter of pAC373, and flanking homologous sequences to direct recombination into the baculovirus genome. A polyadenylation signal, such as the one from the polyhedron gene present in this plasmid vector, may or may not be necessary for expression of the recombinant gene. A marker gene such as the β-galactosidase gene of E. coli, juxtaposed to regulatory sequences including a transcriptional promoter and possibly a polyadenylation signal, may be included in the vector but is not essential for expression of a convected gene.

C.2. Creation of recombinant baculovirus:

A chimeric baculovirus is created by homologous recombination between the expression plasmid containing the PN-1 target gene and wild type baculovirus DNA. Plasmid and wild type baculovirus DNA are co-precipitated by the calcium phosphate technique and added to uninfected Spodoptera frugiperda (Sf9) insect cells. Four to seven days following transfection, cells will exhibit a cytopathic morphology and contain the nuclear occlusion bodies typically produced by viral infection. The cell-free culture media containing both wild type and recombinant virus is harvested.

C.3. Identification and isolation of chimeric baculovirus:

Clonal isolates of virus can be obtained from this co-transfection stock by plaque purification on Sf9 cell monolayers overlaid with agarose. Candidate plaques for analysis will be identified by a plaque morphology negative for occlusion bodies. If the expression plasmid

contains a marker gene such as β-galactosidase, recombinant plaques will be indicated by the blue color produced from a chromogenic substrate such as 5-bromo-4-chloryl-3-indolyl-b-D-galactopyranoside (X-gal) in the agarose plating medium. Picked plaques will be used for inoculation of cells in multiwell dishes. The resulting cell lysates and infected cell supernatants can be evaluated for expression of recombinant PN-1, using standard activity or immunological assays. Positive wells may require additional rounds of plaque purification to obtain pure recombinant virus stocks free from wild type contamination.

C.4. Batch production of PN-1:

Sf9 cells are adapted to growth in serum-free, low protein medium such as ExCell (J.R. Scientific). Cells are collected from suspension culture by gentle centrifugation and resuspended in fresh medium containing the viral inoculum at a concentration of ten million cells per ml., using a multiplicity of infection of one virus plaque forming unit per cell. After a period of two hours, the culture is diluted five fold with fresh medium and incubated two to three days. At the end of that time, the cells are pelleted by centrifugation and the conditioned medium harvested. PN-1 is purified from the cell-free supernatant by standard means.

Variants of PN-1 may be created and produced in the same manner as described above.

C.5. Characterization of insect cell derived PN-1:

PN-1 produced in insect cells using a baculovirus expression system is a glycosylated protein of approximate molecular weight of 42,000 kd. The N-terminal amino acid sequence is identical to that of

mature mammalian cell PN-1, indicating correct processing of the signal sequence. The specific activity vs thrombin and association kinetics, including rate enhancement effect of heparin, are indistinguishable from authentic PN-1.

EXAMPLE D

Production of Recombinant PN-1 Variants in E. coli in Inclusion Bodies or in Soluble Form

D.1 Cloning of PN-1

The cloning of PN-1 and expression has been described (McGrogan, et al., (1988) Bio/Technology). The gene for PN-1 was generated by PCR from the CHO expression vector using the following oligonucleotides:

PNPCR-forward 5' TG.GAA.GGA.CAT.ATG.AAC.TGG.CAT.CTC

PNPCR-reverse 5' TCT.TTT.GTA.TAC.TGA.TCA.GGG.TTT.GT

generating an NdeI and BclI site, respectively. The resulting fragment was cut with NdeI and BclI and subcloned into pGEMEX-1 vector (Promega).

The pGEMEX E. coli expression vector contains three RNA polymerase promoters. The T7 promoter is positioned upstream from the gene 10 leader fragment.

We removed the gene 10 region from pGEMEX, but retained the T7 RNA polymerase binding site and NdeI and BamHI cloning sites. To accomplish this the NdeI site at 3251 in pGEMEX was removed by partial NdeI digest followed by Klenow fill-in and relegation. This plasmid is referred to as pT7-NK. pT7-NK was cut with NdeI and BamHI to remove the gene 10 fusion protein region. The linear vector was isolated and ligated with the PCR-generated PN-1 linear fragment, cut with NdeI and BclI, described above. This plasmid is referred to as pT7PN-1. The correct sequence was confirmed by sequencing the entire coding region for PN-1.

The native signal sequence was removed using PCR and the following oligos:

PCRMET.forward 5'GAT.ATA.<u>CAT.ATG</u>.TCC.CAC.TTC.AAT.CCT.CTG

PCRMET.reverse 5'GGG.GGC.ACT.<u>TGT.CGA.C</u>CC.ACA.CCG.GAA

with an NdeI and SalI site, respectively. This generated a 690 base pair fragment which could replace the native signal and a portion of the amino terminus of PN-1 with a start codon (Met) and the amino terminus of PN-1. Again, the correct sequence was confirmed by sequencing. The expression of the resulting protein is expected to be intracellular, either in inclusion bodies or as soluble protein.

D.2. Mutagenesis of PN-1

The plasmid pT7PN1 has an f1 ori for the production of single-stranded DNA. Thus pT7PN1 was transformed into the E. coli strain CJ236 for the production of ssDNA to be used as a template for site-directed mutagenesis according to the method of Kunkel (Kunkel, T.A. (1988) in Nucleic Acids and Molecular Biology (Eckstein, F., Lilley, D.M.J. Eds.) Vol. 2, p. 124, Springer-Verlag, Berlin and Heidelberg).

The general rationale for mutant generation is based upon four general methods.

In the first method, single amino acid substitutions in the region of P4 to P4′ are generated by site directed mutagenesis. In general, substitutions at the P1 site will have the most dramatic effects. However, substitutions at other residues within the active site region will give changes in association rate constants with serine proteases.

In the second method, sequences found at the active site region of other serpins were grafted onto PN-1. A number of combinations must be created to determine how much of the sequence at the active site

must be changed to change the specificity and kinetics. The P_4 to P_4' region is generally found to be most important, but amino acids residues outside this region can have pronounced affects on protease inhibition.

In the third method, sequences which have been found to be particularly good substrates are added to or used to replace a sequence of PN-1. Prior to making these mutants, it was not clear if these changes would ruin the inhibitory effects of PN-1 and turn PN-1 from an inhibitor of proteolysis into a substrate. In fact, incorporation of Ala-Ala-Pro-Phe, a good substrate sequence for subtilisin, into PN-1 results in a molecule which is cleaved particularly well by subtilisin. However, PN-1 variants have now been obtained which are good inhibitors of mammalian serine proteases based upon this approach.

In the fourth method, optimum inhibitor sequences can be generated by using a phage display system. Since PN-1 forms covalent interactions with the target protease, it is important that one is not selecting for mutants which bind more tightly than the parent PN-1 molecule. Rather, one selects for PN-1 variants which bind more rapidly to the target protease by allowing phage-displayed variant PN-1 library to interact with the immobilized target protease for only short times. Thus, only rapid-binding variants will be selected. This is a novel application of the phage display system.

The following oligonucleotides were used to generate a mutant specific for the protease shown at the end of the sequence:
5'GCA.ATT.CTC.ATT.GCA.NN(G/C).TCA.TCG.CCT.CCC [R345I, R345M, R345L, R345V] (elastase), R345K (plasmin), R345D, R345E 5'ATT.CTC.ATT.GCA.GTG.AGC.TCG.CCT.CCC.TG R345V (elastase)

5'ATT.CTC.ATT.GCA.AGA.ATA.TCG.CCT.CCC.TGG S3461 (Factor Xa)

5'ATT.CTC.ATT.GCA.AGA.ACA.TCG.CCT.CCC.TCC S346T (Factor Xa, C1-esterase)

5'ACA.ACT.GCA.ATT.CTG.GCT.GGA.AGA.TCA.TTG.AAT.CCC.TGG.TTT.ATA I343A;A344G;S347L;P348N (ATIII-like, thrombin, Factor Xa)

5'ACA.ACT.GCA.ATT.CTC.TTT.CCA.AGA.TCA.TCG.CCT.CCC I343F;A344P (FPR, thrombin)

5'ACT.GCA.ATT.CTC.ATT.CCA.TTA.TCA.TCG.CAG.GTC.CGG.TTT.ATA.GTA.GAC A344P;R345L;P348Q;P349V;W350R (HCII-like)

5'GAA.GAT.GGA.ACC.AAA.GCT.TCA.GAC.TTT.TTG.GCT.GAA.GGT.GGC.GGT.GTA.AGA.TCA.TCG.CCT.CCC.TGG A336D;A337F;T338L;T339A;A340E;I341G;L342G;A344V (fibrinogen-like, thrombin)

5'GCA.ACA.ACT.GCA.ATT.ATC.GAG.GGA.AGA.TCA.TCG.CCT L342I;I343E;A344G (Factor Xa)

5'ACA.ACT.GCA.ATT.CTC.GAG.CCA.GTA.TCA.TCG.CCT.CCC I343E;A344P;R345V (elastase, cathepsin G)

5'ACT.GCA.ATT.CTC.ATT.GGA.AGA.TCA.TCG.CCT A344G (faster kinetics)

5'ACT.GCA.ATT.CTC.ATT.CCA.AGA.TCA.TCG.CCT A344P (faster kinetics)

5'GCA.ACA.ACT.GCA.ATT.AGC.CCT.TTC.AGA.TCA.GTG.CAG.CCC TGG.TTT.ATA L342S;I343P;A344F;S347V;P348Q (high molecular weight kininogen-like; kallikrein)

5'GCA.ACA.ACT.GCA.ATT.GCC.GCT.CCA.TTC.TCA.TTG.CCT.CCC.TGG.TTT L342A;I343A;A344P;R345F (cathepsin G)

5'GCA.ACA.ACT.GCA.ATT.GCC.GCT.CCA.GTA.TCA.TCG.CCT.CCC.TGG.TTT L342A;I343A;A344P;R345L (elastase)

5'GCA.ACA.ACT.GCA.ATT.GCC.GCT.CCA.CTA.TCA.TCG.CCT.CCC.TGG.TTT L342A;I343A;A344P;R345L (elastase)

5'GCA.ACA.ACT.GCA.ATT.GCC.GCT.CCA.ATA.TCA.TCG.CCT.CCC.

TGG.TTT L342A;I343A;A344P;R345I (elastase)

D.3. Expression and Purification of Protease Nexin Variants in E. coli

JM109 (DE3) contains a chromosomal copy of the gene which codes for T7RNA polymerase under the control of the inducible lac promoter. JM109 (DE3) containing pT7PN-1 (or a variant of PN-1) was grown overnight in 2xYT + 0.2% glucose + 100 mg/ml carbenicillin at 28-32°C. Low temperature and high nutrient containing solution is helpful in generating productive innoculants. The inoculum was diluted 1:250 to 1:500 and grown to OD_{600}~1 in a shake flask or ~50 in a fermentor at 26-37°C, and induced with IPTG at 0.1-1.0 mM for 4-16 hours. The bacteria were collected by centrifugation, resuspended in 10 mM TRIS, pH 8, 1 mM EDTA, and disrupted by high pressure homogenization. Inclusion bodies were collected by centrifugation, washed with 1 M NaCl, 0.05% triethylamine, and the protein refolded from a 6 M guanidine solution by rapid dilution. PN-1 was purified by capture on FastS sepharose and eluted with 0.6 M NaCl, diluted to 0.25 M NaCl and passed over FastQ sepharose to remove endotoxin and recaptured on FastS sepharose and eluted with 0.6 M NaCl or a gradient of 0.25 to 1 M NaCl.

Alternatively, PN-1 can be generated in a soluble form within E. coli by adjusting the fermentation conditions. This procedure provides a greater yield of soluble PN-1 as the fermentation temperature is decreased from 37°C to 26°C with a concomitant loss in inclusion body material. This is quite an unexpected finding, since PN-1 is bactericidal when native PN-1 is added to E. coli. To purify soluble PN-1, the cell supernatant from the disruption step was clarified by centrifugation and filtration or by treatment with polycations such as polyethyleneimine or Biacryl™ followed by centrifugation

and filtration, and the soluble protein was purified as above. The generation of soluble material has many advantages: there is more certainty that the protein is correctly folded, there are no refolding steps, there is greater reproducibility from batch to batch.

The production of PN-1 was about 50 mg per gram of cell paste. This corresponds to about 50 mg per liter of production at a cell density of 1 OD_{600} or up to 2.5 grams of soluble PN-1 per liter of fermentation. This represents a substantial advance in the state of the art of PN-1 production.

D.4. Activity assay for PN-1 Variants

Refolded or soluble protein was tested for capacity to inhibit thrombin in a standard assay. Briefly, serial 2-fold dilutions of PN-1 variant were added to microtiter plate wells (50 µl/well), followed by 50 µl of a 30 µg/ml heparin solution, followed by 1 NIH unit of thrombin in 50 µl. These were allowed to incubate at 25°C for 15 minutes. Residual thrombin activity was measured by the addition of 50 µl S-2238 (Kabi Pharmaceuticals) at 0.625 mg/ml. PN-1 variants were tested for their ability to inhibit urokinase using the substrate S-2444, plasmin using the substrate S-2390, tPA using the substrate S2288, Factor Xa using the substrate S-2222 or S-2765, kallikrein using the substrate S-2302, human neutrophil elastase using s-AAPV-pna (Sigma), cathepsin G using s-AAPF-pna (Sigma) in a similar manner, with or without the addition of heparin.

The second-order rate association constants were determined for appropriate inhibitors-proteases combinations by combining equal-molar amounts of each protein (determined by titration as above) for various times from 1 second to 4 hours (as appropriate) and

following the activity loss. The $t_{1/2}$ was estimated from resulting curves. The k_{assoc} was estimated according to the equation ln2/ [PN-1] x $t_{1/2}$. Alternatively, the apparent first order rate constant was determined from the slope of a plot of log (normalized activity) vs time. The second order rate constant was calculated by dividing the apparent first order rate constant by the PN-1 (or variant) concentration used.

EXAMPLE E

Generation of ATF-PN1 Chimera

To generate this chimera, we first cloned the amino-terminal fragment of uPA by PCR using the oligonucleotides: ATF.forward
5'GGT.GAT.CAT.ATG.AGC.AAT.GAA.CTT.CAT.CAA
ATF.REVERSE
5'TTT.AGG.ACG.CGT.CTG.CGC.CAT.CTG.CTC.AGT.CAT.G
generating a NdeI and M1uI site respectively. The resulting fragment was cut with NdeI and M1uI and subcloned into pT7PN1 vector cut with the same enzymes to move the signal sequence. This plasmid is referred to as pT7ATF-PN1. The correct sequence was verified by automated sequencing using the T7 dye primer system (ABI).

To generate an even shorter version of ATF-PN1 which retains the urokinase receptor binding region, ATF_{48}-PN1 was made by introducing a M1uI site (underlined) by site directed mutagenesis at codon 48 using the following oligonucleotide:
5'CAC.TGT.GAA.ATA.GAT.AAC.GCG.TAA.ACC.TGC.TAT.GAG.
The resulting plasmid was cut with M1uI to remove a 300 bp segment and ligated.

Mutagenesis of ATF-PN1

The plasmid pT7ATF-PN1 has an f1 ori for the production of single-stranded DNA. Thus pT7ATF-PN1 was transformed into the E. coli strain CJ236 for the production of ssDNA to be used as a template for site-directed mutagenesis according to the method of Kunkel (Kunkel, T.A. (1988) in *Nucleic Acids and Molecular Biology* (Eckstein, F., Lilley, D.M.J. Eds.) Vol. 2, p. 124, Springer-Verlag, Berlin and Heidelberg). In addition, PN-1 variants of interest can be subcloned into pT7ATF-PN-1 by using standard molecular biology techniques.

Expression and Purification of ATF-PN1

The resultant plasmid was transformed into the E. coli strain JM109 (DE3), grown to OD_{600} ~1 in a shake flask or ~50 in a fermentor, and induced with IPTG at 0.1-1.0 mM for 4-16 hours at 26-37°C. The bacteria were collected by centrifugation, resuspended in 10 mM TRIS, pH 8, 1 mM EDTA, and disrupted by high pressure homogenization. Inclusion bodies were collected by centrifugation, washed with 1 M NaCl, 0.05 % TEA, and the protein refolded from a 6 M guanidine solution. ATP-PN1 was purified by capture on FastS sepharose and eluted with 0.6 M NaCl, diluted to 0.25 M NaCl and passed over FastQ sepharose to remove endotoxin and recaptured on FastS sepharose and eluted with 0.6 M NaCl or a gradient of 0.25 to 1 M NaCl. Alternatively, the cell supernatant from the disruption step was clarified by centrifugation and filtration, and the soluble protein was purified as above.

Activity assay for ATF-PN1

Refolded or soluble protein was tested for capacity to inhibit thrombin in a standard assay.

Briefly, serial 2-fold dilutions of ATF-PN1 were added to microtiter plate wells (50 μl/well), followed by 50 μl of a 30 μg/ml heparin solution, followed by 1 NIH unit of thrombin in 50 μl. These are allowed to incubate at 25°C for 15 minutes. Residual thrombin activity is measured by the addition of 50 μl S-2238 (kabi Pharmaceuticals) at 0.625 mg/ml. ATF-PN1 was tested for its ability to inhibit urokinase using the substrate S-2444, or plasmin using the substrate S-2390, in a similar manner, without the addition of heparin.

Refolded or soluble protein was tested for the ability to bind to a soluble form of the urokinase receptor as measured by ELIZA, or the ability to inhibit the binding of urokinase to the soluble urokinase receptor. ATF-PN1 was also tested for its ability to inhibit uPA or DFP/PMFS treated uPA binding to cells such as HT1080, U937, or THP-1 expressing uPA receptor.

EXAMPLE F

Generation of Cysteine-PEGylated Proteins

F.1 Preparation of Maleimido-PEG Reagent

Maleimido-PEG was prepared by mixing the following:

1) 100 mg methoxypolyethylene amine (20 μmol) (MW≈5,000)
2) 20 μmol γ-maleimidobutyric acid-N-hydroxy succinimide ester (GMBS)
3) 2 ml 100 mM Caps buffer, pH 10.0

The amount of the components above (particularly 1) and 2)) and the volume indicated may be varied. For example, it is permissible that the difference in the ratio of methoxypolyethylene amine to GMBS can vary by up to ten to 100-fold. Normally, about a two-fold excess of 1)

above to GMBS is preferred. While various buffers may be substituted for the Caps buffer, it is important that Tris buffers are not used in this mixture, as Tris buffers will quench the reaction. The pH of the buffer used may vary considerably, although buffers having a pH of 10.0 are preferable over buffers having a pH of 8.0. In addition, the mixture above may contain up to 50% DMSO as a cosolvent. It is particularly important that the reaction mixture does not contain a reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol (βME).

The mixture was incubated at 37°C for 30 minutes, although the reaction temperature may be as low as 4°C and the reaction time may be extended for up to one hour or more. After incubation, 12 mg of Tris free base or ethanolamine was added to the mixture to quench the NH_3 moiety. This quenching step may be omitted.

The reacted mixture is purified by elution through a PD-10 column (G-25) (BioRad) equilibrated with 20 mM Tris (pH 7.4), 100 mM NaCl, and 0.1% Tween. The eluant was collected in 0.5 ml fractions and assayed for production of the Maleimido-PEG reagent by precipitation with 50% TCA. The resulting Maleimido-PEG (Mal-PEG) reagent is then used in to modify a selected protein by attachment of PEG to a cysteine residue(s).

F.2 Reaction of Maleimido-PEG with Protein

Prior to reaction of the protein with the Maleimido PEG reagent, the purified protein was diluted to a concentration of about 200 μg/ml to 1 mg/ml in any suitable buffer which does not contain DTT or βME. Normally, the buffer was composed of 20 mM PIPES pH 6.75, 0.6 M NaCl, and 1% glycerol. Approximately 10 μl to 40 μl of the diluted protein was used for the PEGylation reaction. The Maleimido-PEG reagent described in section F.1 was diluted in a series of 2-fold dilutions using

10 μl transfers of solution containing approximately 1 μl Maleimido-PEG in a 10 μl volume of buffer composed of 20 mM Tris pH 7.4, 0.1 M NaCl, and 0.01% Tween. The ratio of the maleimido-PEG to protein may be varied according to the preferred level of PEGylation of the protein desired. Up to 20-fold excess of maleimido-PEG to protein still provided for specific reaction of the reagent with cysteine residues of the protein.

The protein and maleimido-PEG were incubated for one hour at room temperature, although this reaction may be performed at 4°C for longer periods of time. A sample of the reacted mixture may be analyzed by SDS-PAGE to determine the minimal amount of maleimido-PEG reagent needed for complete coupling.

The reaction described above may be used to determine the proper ratio of Maleimido-PEG to protein, and then scaled up to produce commercially acceptable amounts of PEGylated protein.

F.3 Preparation of $(\text{Maleimido})_2$-PEG Reagent

$(\text{Maleimido})_2$-PEG is prepared by mixing the following:

1) polyethylene bis[amine]
2) 20 μmol γ-maleimidobutyric acid-N-hydroxy succinimide ester (GMBS)
3) 2 ml 100 mM Caps buffer, pH 10.0

The amount of the components above (particularly 1) and 2)) and the volume indicated may be varied. For example, it is permissible that the difference in the ratio of 10 and 2) can vary by up to 10 to 100 fold, although an excess of GMBS to 1) above is preferred, normally about a 2-fold excess. While various buffers may be substituted for the Caps buffer, it is important that Tris buffers are not used in this mixture, as Tris buffers will quench

the reaction. The pH of the buffer used may vary considerably, although buffers having a pH of 10.0 are preferable over buffers having a pH of 8.0. In addition, the mixture above may contain up to 50% DMSO as a cosolvent. It is particularly important that the reaction mixture does not contain a reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol (βME).

The mixture is incubated at 37°C for 30 minutes, although the reaction temperature may be as low as 4°C and the reaction time may be extended for up to one hour or more. After incubation, 12 mg of Tris free base or ethanolamine is added to the mixture to quench the NH_3 moiety. This quenching step may be omitted.

The reacted mixture is purified by elution through a PD-10 column (G-25) (BioRad) equilibrated with 20 mM Tris (pH 7.4), 100 mM NaCl, and 0.1% Tween. The eluant is collected in 0.5 ml fractions and production of $(Maleimido)_2$-PEG is assayed by precipitation with 50% TCA. The resulting $(Maleimido)_2$-PEG (Mal-PEG) reagent is then used in to modify a selected protein by attachment of PEG to a cysteine residue(s).

F.4 Reaction of $(Maleimido)_2$-PEG with Protein

Prior to reaction of the protein with the $(Maleimido)_2$-PEG reagent, the purified protein (e.g. a PN-1 mutant containing a cysteine residue at position 99) is diluted to a concentration of about 200 μg/ml to 1 mg/ml in any suitable buffer which does not contain DTT or βME. Normally, the buffer is composed of 20 mM PIPES pH 6.75, 0.6 M NaCl, and 1% glycerol. Approximately 10 μl to 40 μl of the diluted protein is used for the PEGylation reaction. The $(Maleimido)_2$-PEG reagent described in section F.1 is diluted in a series of 2-fold dilutions using 10 μl transfers of solution containing approximately 1 μl $(Maleimido)_2$-PEG in a 10 μl volume of

buffer composed of 20 mM Tris pH 7.4, 0.1 M NaCl, and 0.01% Tween. The ratio of the maleimido-PEG to protein may be varied according to the preferred level of PEGylation of the protein desired.

The protein and $(Maleimido)_2$-PEG are incubated for one hour at room temperature, although this reaction may be performed at 4°C for longer periods of time. A sample of the reacted mixture may be analyzed by SDS-PAGE to determine the minimal amount of $(Maleimido)_2$-PEG reagent needed for complete coupling.

The reaction described above may be used to determine the proper ratio of $(Maleimido)_2$-PEG to protein, and then scaled up to produce commercially acceptable amounts of PEGylated dimeric or multimeric proteins.

EXAMPLE G

Generation of Cysteine-PEGylated PN-1 Variants (Type IV Variants)

G.1 Selection of amino acid residues of PN-1 for substitution by cysteine

PN-1α and PN-1β contain N-glycosylation sites at amino acid residue positions 99 and 140. Therefore, these sites were selected for site-directed mutagenesis to replace the asparagine at one or both of these positions with cysteine.

Three sites in PN-1 were selected for replacement with cysteine on the basis of the presence of glycosylated residues at a corresponding site in a protein homologous to PN-1. Amino acid residue D192 was selected for replacement with cysteine since the proteins angiotensin and Rab ORF1, each which are homologous to PN-1, are N-glycosylated at the amino acids corresponding to this residue in PN-1. Amino acid residue E230 was selected for replacement with cysteine since baboon α_1-antitrypsin (α_1-AT), which is homologous to PN-1, is

glycosylated at the amino acid residue corresponding to this position in PN-1. Amino acid residue H252 was selected for replacement with cysteine since α_2-antiplasmin (α_2-AP), another protein homologous to PN-1, is glycosylated at the corresponding residue.

Other amino acid residues were selected for replacement with cysteine on the basis of the position of the amino acid within the three-dimensional structure of PN-1 as determined by X-ray crystallography (Figure 3). The approximate position of the amino acid residues selected for cysteine substitution are indicated by their corresponding amino acid residue number. The particular amino acid residues identified for mutagenesis in the present example were selected on the basis of the apparent solvent-accessibility of the amino acid and the apparently few number of interactions with other amino acids in the protein.

<u>G.2 Site-directed Mutation of PN-1 to Cysteine</u>

The mutations selected above were generated in PN-1α using site-directed mutagenesis as described in section D.2. Although PN-1α was employed in these experiments, the same mutations in PN-1β are likely to provide the same effects as all the mutations were introduced into the region of amino acid sequence identity between these nearly identical proteins.Briefly, DNA encoding PN-1 was inserted into the plasmid pT7PN1, which has an f1 ori for the production of single-stranded DNA. This plasmid was then transformed into the E. coli strain CJ236 for the production of ssDNA to be used as a template for site-directed mutagenesis according to the method of Kunkel (Kunkel, T.A. (1988) in Nucleic Acids and Molecular Biology (Eckstein, F., Lilley, D.M.J. Eds.) Vol. 2, p. 124, Springer-Verlag, Berlin and Heidelberg).

The oligonucleotides used to generate specific mutations within the PN-1 coding region are as follows:

N140C AAT GCA TGG GTT AAA **AAC** GAA ACC AGG GAT
AAT GCA TGG GTT AAC **TGC** GAA ACC AGG GAT
HpaI

N99C GCC GTG TTT GTT AAG **AAT** GCC TCT GAA ATT
GCC GTG TTT GTT AAC **TGT** GCC TCT GAA ATT
HpaI

P28C GTG AAG TCG AGG **CCT** CAT GAC AAC ATC GTG ATC
GTG AAG TCG AGG **TGC** CAT GAC AAC ATC GTG ATC

G52C CTG GGG GCG GAC TGC AGG ACC AAG AAG
PstI

N85C GTC TCC AAG AAG **AAT** AAA GAC ATT GTG ACA GTG GCT
GTC TCC AAG AAG **TGC** AAA GAT ATC GTG ACA GTG GCT
EcoRV

Q116C AAA GAT GTG TTC **CAG** TGT GAG GTC CGG
AAA GAT GTG TTC TGC AGT GAG GTC CGG
PstI

N304C TCA TCA AAG GCA **AAT** TTT GCA AAA ATA ACA
TCA TCA AAG GCA **TGC** TTT GCA AAA ATA ACA
SphI

S1C GAT ATA CAT ATG TCC CAC TTC AAT **CCT** **CTG** **TCT** CTC GAG
GAT ATA CAT ATG **TGC** CAC TTC AAT CCC TTA AGT CTC GAG
AflII

GAA CTA GGC
GAA CTA GGC

R63C AAG AAG CAG CTC GCC ATG GTG ATG **AGA** TAC GGC GTA AAT
AAG AAG CAG CTC GCA ATG GTG ATG **TGC** TAC GGC GTA AAT
NcoI destroyed

E125C GTC CGG AAT GTG AAC TTT **GAG** GAT CCA GCC TCT
GTC CGG AAT GTT AAC TTT **TGC** GAT CCA GCC TCT
HpaI

D147C AGG GAT ATG ATT **GAC** AAT **CTG** **CTG** **TCC** CCA GAT CTT ATT
AGG GAT ATG ATT **TGC** AAT CTC TTA AGC CCA GAT CTT ATT
AflII

D192C TTC GTG GCA GCA **GAC** GGG AAA TCC TAT
TTC GTG GCA GCA TGC GGG AAA TCC TAT
SphI

E230C CCC TAC CAC GGG **GAA** AGC ATC AGC ATG
CCC TAC CAC GGC TGC AGC ATC AGC ATG
PstI

H252C GCC ATC ATC CCA **CAC** ATC AGC ACC AAG ACC ATA GAC
GCC ATC ATC CCA TGT ATC AGT ACT AAG ACC ATA GAC
ScaI

S263C ACC ATA GAC AGC TGG ATG **AGC** ATG GTC
ACC ATA GAC AGT TGG ATG TGC ATC ATG GTC
PvuII destroyed

P267C AGC ATC ATG GTC **CCC** AAG AGG GTG CAG
AGC ATC ATG GTC TGC AAA CGC GTG CAG
Afl III

D284C GCT GTA GCA CAA ACA **GAT** TTG AAG GAG CCG CTG
GCT GTA GCA CAA ACA TGT TTA AAG GAG CCG CTG
DraI

The mutants are named according to the single-letter code for the amino acid residue in the native protein, the number of the position of that amino acid within the amino acid sequence of PN-1, and the single-letter code for the amino acid residue substituted at that site. For example, the mutant S1C produces a PN-1 protein which has the serine at position 1 replaced by cysteine. The top sequence for each mutant above indicates the wild type PN-1 sequence, while the sequence below indicates the mutation introduced in the coding sequence of the mutant. The nucleotides in bold are changed relative to wild type. The codon which is double-underlined is the newly-introduced codon for cysteine. The underlined sequences in the mutated DNA sequence indicate a restriction enzyme site which is introduced into or removed from the sequence of the mutant. Introduction or removal of these restriction sites do not alter the amino

acid sequence encoded at that site, but provide a means for screening clones containing DNA subjected to site-directed mutagenesis for incorporation of the oligonucleotide sequence into the PN-1 coding sequence. Introduction of the desired mutation was confirmed by restriction enzyme analysis.

Mutant PN-1 proteins containing multiple cysteine-substituted residues were generated by introduction of a first mutation by site-directed mutagenesis as described. After confirmation of the insertion of the first mutation by restriction enzyme analysis, the DNA was subjected to a second round of site-directed mutagenesis using a different oligonucleotide. For example, the double mutant N99C;N140C was generated by site-directed mutagenesis with the N99C oligonucleotide and confirmation of the presence of the newly introduced HpaI site in the coding sequence. The N99C mutant DNA was then subjected to a second round of site-directed mutagenesis with the N140C oligonucleotide. Table G.5A below lists the single, double, and triple mutants generated using these techniques and the oligonucleotides described above.

DNA encoding the mutant PN-1 proteins were expressed and the expressed proteins purified as described in section D.3.

G.3 Reaction of PN-1 and PN-1 Mutants with Maleimido-PEG Reagent

After purification of PN-1 and the PN-1 mutants described above, each protein was reacted with the Maleimido-PEG reagent described in F.1 according to the general protocol of F.2. For example, the mutant N99C;N140C was cysteine-PEGylated using the following protocol.

Purified N99C;N140C protein was diluted to a concentration of about 200 μg/ml in 20 mM PIPES pH 6.75, 0.6 M NaCl, 1% glycerol. Approximately 40 μl of the diluted protein (0.25 nmol) was used for the PEGylation reaction. The Maleimido-PEG reagent described in section F.1 was diluted in a series of 2-fold dilutions using 10 μl transfers starting from approximately 2 μl Maleimido-PEG in a 10 μl volume of buffer composed of 20 mM Tris pH 7.4, 0.1 M NaCl, and 0.01% Tween. This reaction contained a two-fold excess of the Maleimido-PEG reagent over that required for PEGylation of the number of cysteine sites in the PN-1.

The N99C;N140C protein and maleimido-PEG mixtures were incubated for one hour at room temperature. A sample of each of the reacted mixtures was analyzed by SDS-PAGE. Analysis of this gel revealed that the band migrating at the relative molecular weight of unmodified N99C;N140C PN-1 disappeared as the ratio of Maleimido-PEG to protein increased. Accordingly, as the amount of unmodified N99C;N140C PN-1 in the sample disappeared with increasing Maleimido-PEG concentrations, distinct bands migrating at molecular weights of increasing intervals of approximately 5,000 MW appeared. Thus, reaction of the PN-1 variant produced distinct cysteine-PEGylated proteins containing increasing numbers of PEG units per protein molecule, up to 2 PEG per PN-1 molecule, the maximum number of cysteines available in the N99C;N140C PN-1 variant.

Distinct bands representing proteins increasing in relative molecular weight by 5,000 MW intervals is evidence of the specificity of the Maleimido-PEG reaction for attachment of PEG to cysteine residues. If the reaction had resulted in PEGylation of residues other than cysteine, a smear of proteins would appear on the

gel, indicating the presence of proteins containing an infinite number of PEG moieties.

A sample of the cysteine-PEGylated protein which was reacted at a ratio of 2:1 Maleimido-PEG to protein was tested for activity using the assay described in D.4. This sample, which primarily contains cysteine-PEGylated protein, retained at least 100% of the activity of unmodified PN-1. The specific activity of the PEGYlated proteins increased with an increasing amount of the Maleimido-PEG reagent present in the reaction mixture (Figure 4). These data suggest that an increase in activity is often found upon increasing PEG modification, which may result from the increased solubility and/or activity of PEGylated PN-1.

G.4 Generation of PEGylated PN-1 Mutant Using Conventional Method (Comparative Example)

In order to compare the results obtained above with the PEGylation methods known in the art, the N99C;N140C PN-1 variant was PEGylated using a method similar to that described by Zalipsky in USPN 5,122,614, with the substitution of a paranitrophenol carbonate of PEG for the N-succinimide carbonate of PEG used by Zalipsky as the activated carbonate. The protocol used was otherwise identical. Ratios ranging from 1:1 to 100:1 of activated PEG to PN-1 mutant (N99C;N140C) were used in the reactions.

A sample of each of the reacted mixtures containing dilutions of the PEGylation reagent was analyzed by SDS-PAGE. Analysis of this gel revealed that the amount of protein migrating at the molecular weight of the unmodified PN-1 variant decreased with increasing concentrations of the PEGylation reagent of Zalipsky used in the reaction. However, in contrast to the distinct bands generated using the method of the invention, a

smear of proteins of various, increasing molecular weights appeared as the unmodified protein disappeared. The PEGylated proteins produced by the conventional method contained various numbers of PEG moieties per protein molecule, suggesting that attachment of PEG was random and that any number of lysine residues to various positions were modified.

The sample which contained various levels of PEG modification were tested for activity in the assay described in D.4. The data show that as the amount of the Maleimido-PEG reagent present in Maleimido-PEG/protein reaction mixture increased, the specific activity of the protein decreased (Figure 4). This suggests that increasing levels of PEG modification using the conventional method result in a decrease in the activity of the protein.

G.5 Activity of Cysteine-PEGylated PN-1 and PN-1 Mutants

The specific PN-1 mutants and cysteine-PEGylated mutants generated are shown in Table G.5A. Each of the PN-1 site-directed mutant proteins, as well as wild type PN-1, were modified by cysteine-PEGylation using the protocol described in F.2. The activity of wild type PN-1, each of the PN-1 site-directed mutants, and the cysteine-PEGylated wild type and mutant proteins was determined using the assay described in D.4.

TABLE G.5A

	BEFORE PEG MODIFICATION		AFTER PEG MODIFICATION		
MUTANT	SPECIFIC ACTIVITY[1]	REL TO WT[2]	ACTIVITY	REL TO ACTIVITY BEFORE PEG[3]	REL TO WT[2]
WT	0.50	1	0.017	0.03	
N99C	0.20	0.40	0.50	2.5	1.0
N140C	0.050	0.10	0.10	2	0.20
N99C; N140C	0.167	.33	0.33	2	0.75
S1C[5]	0.10	0.20	0.25	2.5	0.50
R63C[5]	0.050	0.10	0.125	2.5	0.25
N85C[5]	0.033	0.066	0.040	1.2	0.080
D147C[5]	0.063	0.125	0.125	2	0.25
D192C[5]	0.045	0.091	0.91	2	0.18
E230C[5]	0.071	0.14	0.20	2.8	0.40
H252C[5]	0.10	0.20	0.25	2.5	0.50
H252C[5]	0.10	0.20	0.25	2.5	0.50
N304C[5]	0.056	0.11	0.17	3	0.33
C117S; C131S; C209S	0.050		0.50		

1 Sp.Act is NIH units of thrombin inhibited per µg PN-1 (variant).

2 Activity relative to wild type is calculated by dividing the activity of wild type PN-1 by the activity of the mutant.

3 Activity of cysteine-PEGylated protein relative to activity of this protein before PEGylation is calculated by dividing the activity of the mutant before PEGylation by the activity of the mutant after PEGylation.

4 Activity of cysteine-PEGylated PN-1 is variable as modification of the naturally occurring Cys 209 inhibits activity.

5 These mutants are in the N99C;N140C mutant background (i.e. these are triple mutants).

The mutations described here for PN-1 can be introduced into any serpin with the expectation of substantially similar effects due to the homology between the members of the serpin protein family.

G.6 Test for Half-Life of Cysteine-PEGylated PN-1 and PN-1 Mutants

The circulating half-life of any protein can be measured by standard methods well known in the art. For example, radioactive PEG-modified protein is injected into a mouse, rat, or rabbit. At various times, blood is withdrawn and the amount of protein remaining in circulation is determined by scintillation counting. Alternatively, PEG-modified PN-1 is injected into a mouse, rat, or rabbit. At various times, blood is withdrawn and urokinase inhibitory activity is measured. In some cases, the amount of protein remaining in circulation can be measured with antibody reaction as in an ELIZA or sandwich ELIZA.

G.7 Administration of Cysteine-PEGylated PN-1

Cysteine-PEGylated PN-1 and/or the cysteine-PEGylated PN-1 mutants described above may be used in the treatment of a variety of disease states for which PN-1 is indicated as therapeutically useful. For example, the proteins may be incorporated into a bandage for dressing a wound as described in USPN 5,196,196, herein incorporated by reference with respect to the use (e.g. dosages and routes of administration) of PN-1 in wound dressings. Alternatively, cysteine-PEGylated PN-1 and/or cysteine-PEGylated PN-1 mutants may be incorporated as the active ingredient(s) in a pharmaceutical compositions

for treatment of inflammation and arthritis, as described in USPN 5,206,017 and USPN 5,326,562, each incorporated herein by reference with respect to the use (e.g. dosages and routes of administration) of PN-1 in treatment of such conditions.

EXAMPLE H

Generation of Cysteine-PEGylated Erythropoietin (EPO)

H.1 Selection of Amino Acid Residues for Cysteine Substitution

The amino acid sequence of erythropoietin (EPO) is as follows:

*MGVHECPAWLWLLLSLLSLPLGLPVLG*APPRLICDSRVLQRYLLEAKEAE 50
NITTGCAEHCSLNE**N**ITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEA 100
VLRGQALLV**N**SSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPD 150
AASAAPLRTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR 194

The first 27 amino acids of the protein (italicized) are the EPO signal sequence. The amino acid residues which are in bold and underlined above (N24, N38, and N83) are sites of N-glycosylation in the native EPO protein. These sites are thus selected for replacement with a cysteine residue, which is subsequently modified by PEGylation.

H.2 Site-directed Mutagenesis of EPO

The complete nucleotide sequence which codes for the mature EPO protein is known in the art and available from GenBank. DNA encoding the mature EPO protein is cloned and subjected to site-directed mutagenesis as described in D.2. Oligonucleotides for replacement of residues N24, N38, and N83 with cysteine are as follows:

N24C: GCC AAG GAG GCC GAG **<u>TG</u>**T ATC ACG ACG GGC
N38C: TGC AGC TTG AAT GAG **<u>TG</u>**T ATC ACT GTC CCA
N83C: GCC CTG TTG GTC **<u>TG</u>**C TCT TCC CAG CCG

The residues in bold and underlined indicate the nucleotides which are different relative to the wild type EPO DNA sequence and represent the cysteine codon to be introduced into the EPO amino acid sequence.

The mutant EPO proteins which contain a cysteine residue at N24, N38, N83, or a combination of these site (e.g. double and triple mutants) are generated as described, expressed, and purified using techniques well known in the art.

<u>H.3 Generation of Cysteine-PEGylated EPO and Cysteine-PEGylated EPO Mutants</u>

Purified EPO mutants N24C, N38C, N83C, and mutants containing combinations of these mutations are subjected to cysteine-PEGylation using the protocol described in F.2. Samples of the reacted proteins are analyzed by SDS-PAGE to determine the extent of the PEGylation, as well as the minimum amount of the Maleimido-PEG reagent necessary to produce fully PEGylated protein.

The activity of the cysteine-PEGylated wild type EPO, as well as the cysteine-PEGylated EPO mutants are tested using protocols known in the art.

<u>EXAMPLE I</u>

<u>Generation of Cysteine-PEGylated Human Growth Hormone (hGH)</u>

<u>I.1 Selection of Amino Acid Residues for Cysteine Substitution</u>

The nucleotide sequence, amino acid sequence, and the three-dimensional structure of human growth hormone

(hGH) are well known in the art (see, for example, Cunningham and Wells 1989 *Science* **244**:1081-1085). In addition, the three-dimensional structure of hGH bound to its receptor is known (De Vos et al. 1992 *Science* **255**:306-312). Amino acid residues for replacement with cysteine are selected based upon the solvent-accessibility of the amino acid residue, the proximity of the residue to other amino acid residues with which it may interact, and the distance of the residue from regions of hGH which are known to be important for receptor binding (Cunningham and Wells 1989 *Science* **244**:1081-1085).

I.2 Site-directed Mutagenesis of hGH

Oligonucleotides for site-directed mutagenesis are designed so as to introduce a cysteine residue in place of the amino acid residue(s) selected above. Site-directed mutagenesis is performed as described in D.2. The resulting hGH DNA is then inserted into an expression vector, and the resultant protein is expressed in *E. coli* or other suitable host. The resulting hGH mutant protein is then purified according to methods known in the art.

I.3 Generation of Cysteine-PEGylated hGH

The hGH mutant protein is then subjected to cysteine-PEGylation using the method outlined in F.2. A sample of a reacted mixture of Maleimido-PEG and hGH mutant protein is analyzed by SDS-PAGE to determine the optimal conditions for cysteine-PEGylation (e.g. the minimal amount of the Maleimido-PEG reagent necessary to provide the desired PEGylated hGH mutant protein).

The cysteine-PEgylated hGH protein is then tested for activity by assaying for the ability of the modified protein to bind to purified, truncated hGH receptor, as described in Cunningham and Wells (*ibid.*).

EXAMPLE J

Generation of Hemoglobin by Cysteine-PEGylation Cross-Linking

Hemoglobin is a tetrameric protein complex composed of two "a" chains and two "b" chains. The amino acid sequences of the "a" and "b" chains of hemoglobin, as well as the tetrameric complex of hemoglobin composed of 2 "a" and 2 "b" chains are well known. Appropriate amino acid residues which are solvent-accessible and minimally contacted with other side chains are selected for site-directed mutagenesis to cysteine. The "a" and "b" chain mutants are then expressed, purified, and allowed to form a tetramer. The mutant tetrameric complex is then reacted with various levels of $(Maleimido)_2$-PEG as described in F.4 above. This reaction can be carried out with very dilute hemoglobin levels to form intramolecular cross-links to stabilize the tetrameric form of hemoglobin, with a minimum number of intermolecular cross-links. Alternatively, the reaction can be carried out a higher with a higher hemoglobin concentration, resulting in higher levels of intermolecular cross-linking to stabilize an aggregate of hemoglobin molecules.

While the present invention has been described with reference to specific protease nexin-1 variants and formulations containing such, it should be understood by those skilled in the art that various changes may be made and equivalence may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, excipient, PN-1 variant, process, process step or steps to the objective, spirit and scope of the invention. All such modifications are intended to be within the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

CLAIMS:

1. A protease nexin-1 variant wherein an amino acid residue at a position selected from the group consisting of P4, P3, P2, P1, P1', P2', P3', P4' is replaced with a natural amino acid residue which is different from the amino acid residue naturally present at that position.

2. The protease nexin-1 variant of claim 1, wherein the variant has a different protease specificity as compared with protease nexin-1 and/or an increased rate association constant with respect to a specific protease as compared with protease nexin-1.

3. A protease nexin-1 variant wherein amino acid residues at the active site of protease nexin-1 are replaced with an equivalent number of active site amino acid residues of a serine protease inhibitor other than protease nexin-1.

4. The variant of claim 3, wherein the serine protease inhibitor is selected from the group consisting of antithrombin III, heparin cofactor II, α-1-antitrypsin, α-1-protease inhibitor, plasminogen activator inhibitor I, II, & III, α-2-antiplasmin, kallikrein-binding protein, and C1-inhibitor.

5. The variant of claim 3, wherein amino acid residues at positions P4, P3, P2, P1, P1', P2', P3', P4' of the active site of protease nexin-1 are replaced with an amino acid sequence selected from the group consisting of:

P_4	P_3	P2	P_1	P_1'	P_2'	P_3'	P_4'
Val-	Ser-	Ala-	Arg	Met-	Ala-	Pro-	Glu
Met-	Thr-	Gly-	Arg	Thr-	Gly-	His-	Gly
Phe-	Thr-	Phe-	Arg	Ser-	Ala-	Arg-	Leu
Ile-	Ala-	Gly-	Arg	Ser-	Leu-	Asn-	Pro

11	Ala- Met- Ser- Arg	Met- Ser- Leu- Ser
12	Ser- Val- Ala- Arg	Thr- Leu- Leu- Val
13	Ile- Leu- Ser- Arg	Arg- Thr- Ser- Leu
14	Phe- Arg- Ile- Leu	Ser- Arg- Arg- Thr
15	Ala- Ile- Pro- Met	Ser- Ile- Pro- Pro
16	Glu- Lys- Ala- Trp	Ser- Lys- Tyr- Gln
17	Leu- Leu- Ser- Ala	Leu- Val- Glu- Thr
18	Ile- Thr- Leu- Leu	Ser- Ala- Leu- Val
19	Phe- Met- Pro- Leu	Ser- Thr- Glu- Val
20	Met- Thr- Gly- Arg	Thr- Gly- His- Gly.

6. A protease nexin-1 variant wherein three or more amino acid residues of the active site of protease nexin-1 are replaced with different amino acid residues which comprise a substrate sequence specific for a given protease.

7. The variant of claim 6, wherein the given protease is selected from the group consisting of elastase, cathepsin G, C1-esterase, thrombin, kallikrein, and Factor Xa, Factor IXa, Factor XIa, Factor XIIa, Factor VIIIa, Factor V', Activated Protein C, trypsin, chymotrypsin.

8. A protein or portion thereof containing three or more amino acids, at least one of which is cysteine, wherein polyethylene glycol is covalently bound to a thio group of the cysteine.

9. A modified protein comprised of the amino acid sequence of a naturally occurring protein which sequence includes at least one cysteine residue wherein the modification comprises the coupling of polyethylene glycol to a cysteine residue of the protein.

10. A method of coupling polyethylene glycol to a protein comprising the steps of:

identifying a protein of interest and determining a site for coupling of polyethylene glycol to the protein;

coupling polyethylene glycol to the protein at said site wherein the polyethylene glycol is covalently bound to a thio group of a cysteine residue of the protein.

11. The method of claim 10, wherein the cysteine residue is naturally present in the protein.

12. The method of claim 10, wherein the protein is altered to include a cysteine residue not normally present and the polyethylene glycol is covalently bound to the added cysteine residue.

13. The method of claim 12 wherein the site for coupling of polyethylene glycol is within a solvent accessible region of the protein.

14. The method of claim 12 wherein the protein is altered to include the cysteine residue at a site of glycosylation.

15. The method of claim 12, wherein the protein is altered to include multiple cysteine residues.

16. The method of claim 10 wherein said polyethylene glycol comprises at least two protein-reactive moieties.

17. The method of claim 10, wherein said polyethylene glycol is between 200 and 10,000 molecular weight.

18. The method of claim 10, wherein the protein of interest is protease nexin-1.

19. The method of claim 10, wherein the protein of interest is a protease nexin-1 variant, said variant having an amino acid residue at a position selected from the group consisting of P_4, P_3, P_2, P_1, P_1', P_2', P_3' and P_4' replaced with a natural amino acid residue which is different from the amino acid residue naturally present at that position.

20. The method of claim 10, wherein the protein of interest is a protease nexin-1 variant wherein at least one amino acid residue at the active site of protease nexin-1 is replaced with an equivalent number of active site amino acid residues of a serine protease inhibitor other than protease nexin-1.

21. The method of claim 10, wherein the protein of interest is a protease nexin-1 variant wherein three or more amino acid residues of the active site of protease nexin-1 are replaced with different amino acid residues which comprise a substrate sequence specific for a given protease.

22. A modified protease nexin-1 protein comprised of the amino acid sequence of naturally occurring protease nexin-1 protein which sequence includes at least one cysteine residue, wherein the modification comprises the coupling of polyethylene glycol to a cysteine residue of the protein.

23. A modified protease nexin-1 variant comprised of the amino acid sequence of protease nexin-1, wherein an amino acid residue at a position selected from the group consisting of P_4, P_3, P_2, P_1, P_1', P_2', P_3' and P_4' is replaced with a natural amino acid residue which is different from the amino acid residue naturally present at that position, and which sequence includes at least one cysteine residue, wherein the modification comprises the coupling of polyethylene glycol to a cysteine residue of the protein.

24. The modified protease nexin-1 variant protein of claim 23, wherein the variant has a different protease specificity as compared with protease nexin-1 and/or an increased rate association constant with respect to a specific protease as compared with protease nexin-1.

25. A modified protease nexin-1 variant wherein amino acid residues at the active site of protease nexin-1 are replaced with an equivalent number of active site amino acid residues of a serine protease inhibitor other than protease nexin-1, and the amino acid sequence of the variant protein includes at least one cysteine residue, wherein the modification comprises the coupling of polyethylene glycol to a cysteine residue of the protein.

26. The modified variant of claim 25, wherein the serine protease inhibitor is selected from the group consisting of antithrombin III, heparin cofactor II, α-1-protease inhibitor, plasminogen activator inhibitor I, II, & III, α-2-antiplasmin, kallikrein-binding protein, and C1-inhibitor.

27. The modified variant of claim 25, wherein amino acid residues at positions P_4, P_3, P_2, P_1, P_1', P_2', P_3' and P_4' of the active site of protease nexin-1 are replaced with an amino acid sequence selected from the group consisting of:

P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'
Val-	Ser-	Ala-	Arg	Met-	Ala-	Pro-	Glu
Met-	Thr-	Gly-	Arg	Thr-	Gly-	His-	Gly
Phe-	Thr-	Phe-	Arg	Ser-	Ala-	Arg-	Leu
Ile-	Ala-	Gly-	Arg	Ser-	Leu-	Asn-	Pro
Ala-	Met-	Ser-	Arg	Met-	Ser-	Leu-	Ser
Ser-	Val-	Ala-	Arg	Thr-	Leu-	Leu-	Val
Ile-	Leu-	Ser-	Arg	Arg-	Thr-	Ser-	Leu
Phe-	Arg-	Ile-	Leu	Ser-	Arg-	Arg-	Thr
Ala-	Ile-	Pro-	Met	Ser-	Ile-	Pro-	Pro
Glu-	Lys-	Ala-	Trp	Ser-	Lys-	Tyr-	Gln
Leu-	Leu-	Ser-	Ala	Leu-	Val-	Glu-	Thr
Ile-	Thr-	Leu-	Leu	Ser-	Ala-	Leu-	Val
Phe-	Met-	Pro-	Leu	Ser-	Thr-	Glu-	Val
Met-	Thr-	Gly-	Arg	Thr-	Gly-	His-	Gly

and the variant protein contains at least one cysteine residue, wherein the modification comprises the coupling of polyethylene glycol to a cysteine residue of the protein.

28. A modified protease nexin-1 variant wherein three or more amino acid residues of the active site of protease nexin-1 are replaced with different amino acid residues which comprise a substrate sequence specific for a given protease and the amino acid sequence of the variant protein includes at least one cysteine residue wherein the modification comprises the coupling of polyethylene glycol to a cysteine residue of the protein.

29. The modified protease nexin-1 variant of claim 28, wherein the given protease is selected from the group consisting of elastase, cathepsin G, C1-esterase, thrombin, kallikrein, Factor Xa, Factor IXa, Factor XXIa, Factor VIIIa, Factor V', Activated Protein C, trypsin, and chymotrypsin.

30. A compound having the following general structural formula:

$$R_1\text{-S-PEG-S-}R_2$$

wherein R_1 and R_2 are independently each an amino acid sequence, each S is a thio group of a cysteine residue of each of R_1 and R_2, and PEG is polyethylene glycol.

31. The compound of claim 30, wherein R_1 and R_2 each independently comprise from about 6 to 1,000 amino acids.

32. The compound of claim 30, wherein said polyethylene glycol is from 200 to 10,000 molecular weight.

33. The compound of claim 30, wherein in R_1 and R_2 are the same.

34. The compound of claim 30, wherein R_1 is hemoglobin a chain and R_2 is hemoglobin b chain.

35. A compound having the following general formula:

$$R_1 - (S - PEG - S - R_2)_n$$

wherein R_1 and R_2 are independently each amino acid sequences, S is a thio group of a cysteine residue of each of R_1 and R_2, and PEG is polyethylene glycol.

36. The compound of claim 35, wherein said polyethylene glycol is from 200 to 10,000 molecular weight.

37. The compound of claim 35, wherein R_1 and R_2 are the same.

38. The compound of claim 37, wherein R_1 is hemoglobin a chain and R_2 is hemoglobin b chain.

39. A DNA sequence encoding the protease nexin-1 variant of claim 1.

40. A DNA sequence encoding the protease nexin-1 variant of claim 2.

41. A DNA sequence encoding the protease nexin-1 variant of claim 4.

42. A DNA sequence encoding the protease nexin-1 variant of claim 5.

43. A DNA sequence encoding the chimeric protease of claim 8.

44. A pharmaceutical composition, comprising:
a pharmaceutically acceptable carrier; and
a variant of protease nexin-1 as claimed in claim 1.

45. A pharmaceutical composition, comprising:
a pharmaceutically acceptable carrier; and
a variant of protease nexin-1 as claimed in claim 2.

46. A pharmaceutical composition, comprising:
a pharmaceutically acceptable carrier; and
a variant of protease nexin-1 as claimed in claim 4.

47. A pharmaceutical composition, comprising:
a pharmaceutically acceptable carrier; and
a variant of protease nexin-1 as claimed in claim 5.

48. A method of producing a variant protein, comprising:
connecting DNA encoding amino acids of a receptor binding region of a first naturally occurring protein with DNA encoding amino acids of a second protein or a biologically active portion thereof which is different from the first protein; and
expressing the DNA in a suitable host.

49. The variant of claim 48, wherein the second protein is a variant of PN-1 wherein an amino acid residue at a position selected from the group consisting of P4, P3, P2, P1, P1', P2', P3', P4' is replaced with a natural amino acid residue which is different from the amino acid residue naturally present at that position.

50. A method as in claim 48 wherein the receptor binding region is from a first protein selected from the group consisting of urokinase, tPA, Factor IX, Factor X, Protein C, Epidermal growth factor and EGF-like domains.

51. A method as in claim 50 wherein the receptor-binding region is an amino terminal fragment of urokinase.

52. A method as in claim 51 where the receptor-binding region is an amino terminal fragment of urokinase including amino acids 1-135 or 1-87.

53. A pharmaceutical composition, comprising:

a pharmaceutically acceptable carrier; and

a protein or portion thereof as claimed in claim 8.

SEQUENCE OF PROTEASE NEXIN I TYPE ALPHA

```
CTGTGACCCTCCTCGCCGCCGCCGCTTCGCTCGCTCCTCCGACTCCCCCGCCGCCGAGACTAGGCTCCGCTCCGGTTGCGGCGACCCCTCCGCGGCCGCCCCTGGGGATCCAGCGAGCG

                          S1                                          S10                                             1
CGGTCGTCCTTGGTGGAAGGAACC  ATG AAC TGG CAT CTC CCC CTC TTC CTC TTG GCC TCT GTG ACG CTG CCT TCC ATC TGC TCC CAC TTC AAT
                          Met Asn Trp His Leu Pro Leu Phe Leu Leu Ala Ser Val Thr Leu Pro Ser Ile Cys Ser His Phe Asn

                     10                                     20                                            30
CCT CTG TCT CTC GAG GAA CTA GGC TCC AAC ACG GGG ATC CAG GTT TTC AAT CAG ATT GTG AAG TCG AGG CCT CAT GAC AAC ATC GTG ATC
Pro Leu Ser Leu Glu Glu Leu Gly Ser Asn Thr Gly Ile Gln Val Phe Asn Gln Ile Val Lys Ser Arg Pro His Asp Asn Ile Val Ile

                     40                                     50                                            60
TCT CCC CAT GGG ATT GCG TCG GTC CTG GGG ATG CTT CAG CTG GGG GCG GAC GGC AGG ACC AAG AAG CAG CTC GCC ATG GTG ATG AGA TAC
Ser Pro His Gly Ile Ala Ser Val Leu Gly Met Leu Gln Leu Gly Ala Asp Gly Arg Thr Lys Lys Gln Leu Ala Met Val Met Arg Tyr

                     70                                     80                                            90
GGC GTA AAT GGA GTT GGT AAA ATA TTA AAG AAG ATC AAC AAG GCC ATC GTC TCC AAG AAG AAT AAA GAC ATT GTG ACA GTG GCT AAC GCC
Gly Val Asn Gly Val Gly Lys Ile Leu Lys Lys Ile Asn Lys Ala Ile Val Ser Lys Lys Asn Lys Asp Ile Val Thr Val Ala Asn Ala

                     100                                    110                                           120
GTG TTT GTT AAG AAT GCC TCT GAA ATT GAA GTG CCT TTT GTT ACA AGG AAC AAA GAT GTG TTC CAG TGT GAG GTC CGG AAT GTG AAC TTT
Val Phe Val Lys Asn Ala Ser Glu Ile Glu Val Pro Phe Val Thr Arg Asn Lys Asp Val Phe Gln Cys Glu Val Arg Asn Val Asn Phe

                     130                                    140                                           150         BglII
GAG GAT CCA GCC TCT GCC TGT GAT TCC ATC AAT GCA TGG GTT AAA AAT GAA ACC AGG GAT ATG ATT GAC AAT CTG CTG TCC CCA GAT CTT
Glu Asp Pro Ala Ser Ala Cys Asp Ser Ile Asn Ala Trp Val Lys Asn Glu Thr Arg Asp Met Ile Asp Asn Leu Leu Ser Pro Asp Leu

                     160                                    170                                           180
ATT GAT GGT GTG CTC ACC AGA CTG GTC CTC GTC AAC GCA GTG TAT TTC AAG GGT CTG TGG AAA TCA CGG TTC CAA CCC GAG AAC ACA AAG
Ile Asp Gly Val Leu Thr Arg Leu Val Leu Val Asn Ala Val Tyr Phe Lys Gly Leu Trp Lys Ser Arg Phe Gln Pro Glu Asn Thr Lys

                     190                                    200                                           210 SalI
AAA CGC ACT TTC GTG GCA GCC GAC GGG AAA TCC TAT CAA GTG CCA ATG CTG GCC CAG CTC TCC GTG TTC CGG TGT GGG TCG ACA AGT GCC
Lys Arg Thr Phe Val Ala Ala Asp Gly Lys Ser Tyr Gln Val Pro Met Leu Ala Gln Leu Ser Val Phe Arg Cys Gly Ser Thr Ser Ala

                     220                                    230                                           240     SacI
CCC AAT GAT TTA TGG TAC AAC TTC ATT GAA CTG CCC TAC CAC GGG GAA AGC ATC AGC ATG CTG ATT GCA CTG CCG ACT GAG AGC TCC ACT
Pro Asn Asp Leu Trp Tyr Asn Phe Ile Glu Leu Pro Tyr His Gly Glu Ser Ile Ser Met Leu Ile Ala Leu Pro Thr Glu Ser Ser Thr
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FIG. 1-1

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                    250                                                     260                                                     270
CCG CTG TCT GCC ATC ATC CCA CAC ATC AGC ACC AAG ACC ATA GAC AGC TGG ATG AGC ATC ATG GTG CCC AAG AGG GTG CAG GTG ATC CTG
Pro Leu Ser Ala Ile Ile Pro His Ile Ser Thr Lys Thr Ile Asp Ser Trp Met Ser Ile Met Val Pro Lys Arg Val Gln Val Ile Leu

                    280                                     290                                     300
CCC AAG TTC ACA GCT GTA GCA CAA ACA GAT TTG AAG GAG CCG CTG AAA GTT CTT GGC ATT ACT GAC ATG TTT GAT TCA TCA AAG GCA AAT
Pro Lys Phe Thr Ala Val Ala Gln Thr Asp Leu Lys Glu Pro Leu Lys Val Leu Gly Ile Thr Asp Met Phe Asp Ser Ser Lys Ala Asn

                    310                                     320                                     330             HindIII
TTT GCA AAA ATA ACA AGG TCA GAA AAC CTC CAT GTT TCT CAT ATC TTG CAA AAA GCA AAA ATT GAA GTC AGT GAA GAT GGA ACC AAA GCT
Phe Ala Lys Ile Thr Arg Ser Glu Asn Leu His Val Ser His Ile Leu Gln Lys Ala Lys Ile Glu Val Ser Glu Asp Gly Thr Lys Ala

                    340                                     350                                     360
TCA GCA GCA ACA ACT GCA ATT CTC ATT GCA AGA TCA TCG CCT CCC TGG TTT ATA GTA GAC AGA CCT TTT CTG TTT TTC ATC CGA CAT AAT
Ser Ala Ala Thr Thr Ala Ile Leu Ile Ala Arg Ser Ser Pro Pro Trp Phe Ile Val Asp Arg Pro Phe Leu Phe Phe Ile Arg His Asn

                    370                             378
CCT ACA-GGT GCT GTG TTA TTC ATG GGG CAG ATA AAC AAA CCC TGA AGAGTATACAAAAGAAACCATGCAAAGCAACGACTACTTTGCTACGAAGAAAGACTCCT
Pro Thr Gly Ala Val Leu Phe Met Gly Gln Ile Asn Lys Pro ---

TTCCTGCATCTTTCTGTTAAATATTCTTGTACATCGCATTCTTTTTCAAAACGTAGTTTCTTAGGAAGCAGACTCGATGCAACTGTTCCTGTTCTGGGAGGTATTGGAGGGAAAAAACA

AGCAGGATGCCTGGCACAGCTGTACTGAGGATTGATATAGAAAGACTTCCAGATGCCTAAAAGATTCTTTAAACTACTGAACTGTTACCTAGGTTAACATCCCTGTTGAGGTATTTGCT

GTTTGTCCAGTTAGGAATTTTTGTTTTGTTTTGCTCTATATGTGCGGCTTTTCAGAAGAAATTTAATCAGTGTGACAGAAAAAAAAAATGTTTTATGGTAGCTTTTACTTTTTATGAAA

AAAAAATTATTTGTTCCTTTTAAATTCTTTTCCCCCATCCCCCTCCAAAGTCTTGATAGCAAGCGTTATTTTGGGGGTAGAAACGGTGAAATCTCTAGCCTCTTTGTGTTTTTGTTGTT

GTTGTTGTTGTTGTTTTATATAATGCATGTATTCACTAAAATAAAATTTAAAAAACGTCCTGTCTTGCTAGACAAGGTTGTGCATGTGCCTGTCACTACTGAGTCTGTCTACCTATGGA

TTTGCATTTTTGTATTTTGTACAAAGTAAAAATAACT
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FIG. 1-2

SEQUENCE OF PROTEASE NEXIN I TYPE BETA

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CTGTGACCCTCCTCGCCGCCGCCGCTTCGCTCGCTCCTCCGACTCCCCGCCGCCGAGACTAGGCTCCGCTCCGGTTGCGGCGACCCCTCCGCGGCCGCCCCTGGGGATCCAGCGAGCG

                           S1                                          S10                                                     1
CGGTCGTCCTTGGTGGAAGGAACC   ATG AAC TGG CAT CTC CCC CTC TTC CTC TTG GCC TCT GTG ACG CTG CCT TCC ATC TGC TCC CAC TTC AAT
                           Met Asn Trp His Leu Pro Leu Phe Leu Leu Ala Ser Val Thr Leu Pro Ser Ile Cys Ser His Phe Asn

                    10                                      20                                      30
CCT CTG TCT CTC GAG GAA CTA GGC TCC AAC ACG GGG ATC CAG GTT TTC AAT CAG ATT GTG AAG TCG AGG CCT CAT GAC AAC ATC GTG ATC
Pro Leu Ser Leu Glu Glu Leu Gly Ser Asn Thr Gly Ile Gln Val Phe Asn Gln Ile Val Lys Ser Arg Pro His Asp Asn Ile Val Ile

                    40                                      50                                      60
TCT CCC CAT GGG ATT GCG TCG GTC CTG GGG ATG CTT CAG CTG GGG GCG GAC GGC AGG ACC AAG AAG CAG CTC GCC ATG GTG ATG AGA TAC
Ser Pro His Gly Ile Ala Ser Val Leu Gly Met Leu Gln Leu Gly Ala Asp Gly Arg Thr Lys Lys Gln Leu Ala Met Val Met Arg Tyr

                    70                                      80                                      90
GGC GTA AAT GGA GTT GGT AAA ATA TTA AAG AAG ATC AAC AAG GCC ATC GTC TCC AAG AAG AAT AAA GAC ATT GTG ACA GTG GCT AAC GCC
Gly Val Asn Gly Val Gly Lys Ile Leu Lys Lys Ile Asn Lys Ala Ile Val Ser Lys Lys Asn Lys Asp Ile Val Thr Val Ala Asn Ala

                    100                                     110                                     120
GTG TTT GTT AAG AAT GCC TCT GAA ATT GAA GTG CCT TTT GTT ACA AGG AAC AAA GAT GTG TTC CAG TGT GAG GTC CGG AAT GTG AAC TTT
Val Phe Val Lys Asn Ala Ser Glu Ile Glu Val Pro Phe Val Thr Arg Asn Lys Asp Val Phe Gln Cys Glu Val Arg Asn Val Asn Phe

                    130                                     140                                     150         BglII
GAG GAT CCA GCC TCT GCC TGT GAT TCC ATC AAT GCA TGG GTT AAA AAT GAA ACC AGG GAT ATG ATT GAC AAT CTG CTG TCC CCA GAT CTT
Glu Asp Pro Ala Ser Ala Cys Asp Ser Ile Asn Ala Trp Val Lys Asn Glu Thr Arg Asp Met Ile Asp Asn Leu Leu Ser Pro Asp Leu

                    160                                     170                                     180
ATT GAT GGT GTG CTC ACC AGA CTG GTC CTC GTC AAC GCA GTG TAT TTC AAG GGT CTG TGG AAA TCA CGG TTC CAA CCC GAG AAC ACA AAG
Ile Asp Gly Val Leu Thr Arg Leu Val Leu Val Asn Ala Val Tyr Phe Lys Gly Leu Trp Lys Ser Arg Phe Gln Pro Glu Asn Thr Lys

                    190                                     200                                     210 SalI
AAA CGC ACT TTC GTG GCA GCC GAC GGG AAA TCC TAT CAA GTG CCA ATG CTG GCC CAG CTC TCC GTG TTC CGG TGT GGG TCG ACA AGT GCC
Lys Arg Thr Phe Val Ala Ala Asp Gly Lys Ser Tyr Gln Val Pro Met Leu Ala Gln Leu Ser Val Phe Arg Cys Gly Ser Thr Ser Ala

                    220                                     230                                     240     SacI
CCC AAT GAT TTA TGG TAC AAC TTC ATT GAA CTG CCC TAC CAC GGG GAA AGC ATC AGC ATG CTG ATT GCA CTG CCG ACT GAG AGC TCC ACT
Pro Asn Asp Leu Trp Tyr Asn Phe Ile Glu Leu Pro Tyr His Gly Glu Ser Ile Ser Met Leu Ile Ala Leu Pro Thr Glu Ser Ser Thr
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FIG. 2-1

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                250                                         260                                         270
CCG CTG TCT GCC ATC ATC CCA CAC ATC AGC ACC AAG ACC ATA GAC AGC TGG ATG AGC ATC ATG GTG CCC AAG AGG GTG CAG GTG ATC CTG
Pro Leu Ser Ala Ile Ile Pro His Ile Ser Thr Lys Thr Ile Asp Ser Trp Met Ser Ile Met Val Pro Lys Arg Val Gln Val Ile Leu

                280                                         290                                         300
CCC AAG TTC ACA GCT GTA GCA CAA ACA GAT TTG AAG GAG CCG CTG AAA GTT CTT GGC ATT ACT GAC ATG TTT GAT TCA TCA AAG GCA AAT
Pro Lys Phe Thr Ala Val Ala Gln Thr Asp Leu Lys Glu Pro Leu Lys Val Leu Gly Ile Thr Asp Met Phe Asp Ser Ser Lys Ala Asn

                310                                         320                                         330         HindIII
TTT GCA AAA ATA ACA ACA GGG TCA GAA AAC CTC CAT GTT TCT CAT ATC TTG CAA AAA GCA AAA ATT GAA GTC AGT GAA GAT GGA ACC AAA
Phe Ala Lys Ile Thr Thr Gly Ser Glu Asn Leu His Val Ser His Ile Leu Gln Lys Ala Lys Ile Glu Val Ser Glu Asp Gly Thr Lys

                340                                         350                                         360
GCT TCA GCA GCA ACA ACT GCA ATT CTC ATT GCA AGA TCA TCG CCT CCC TGG TTT ATA GTA GAC AGA CCT TTT CTG TTT TTC ATC CGA CAT
Ala Ser Ala Ala Thr Thr Ala Ile Leu Ile Ala Arg Ser Ser Pro Pro Trp Phe Ile Val Asp Arg Pro Phe Leu Phe Phe Ile Arg His

                370                             378
AAT CCT ACA GGT GCT GTG TTA TTC ATG GGG CAG ATA AAC AAA CCC TGA AGAGTATACAAAAGAAACCATGCAAAGCAACGACTACTTTGCTACGAAGAAAGACT
Asn Pro Thr Gly Ala Val Leu Phe Met Gly Gln Ile Asn Lys Pro ---

CCTTTCCTGCATCTTTCTGTTAAATATTCTTGTACATCGCATTCTTTTTCAAAACGTAGTTTCTTAGGAAGCAGACTCGATGCAACTGTTCCTGTTCTGGGAGGTATTGGAGGGAAAAA

ACAAGCAGGATGCCTGGCACAGCTGTACTGAGGATTGATATAGAAAGACTTCCAGATGCCTAAAAGATTCTTTAAACTACTGAACTGTTACCTAGGTTAACATCCCTGTTGAGGTATTT

GCTGTTTGTCCAGTTAGGAATTTTTGTTTTGTTTTGCTCTATATGTGCGGCTTTTCAGAAGAAATTTAATCAGTGTGACAGAAAAAAAAAAATGTTTTATGGTAGCTTTTACTTTTTATG

AAAAAAAAATTATTTGTTCCTTTTAAATTCTTTTCCCCCATCCCCCTCCAAAGTCTTGATAGCAAGCGTTATTTTGGGGGTAGAAACGGTGAAATCTCTAGCCTCTTTGTGTTTTTGTT

GTTGTTGTTGTTGTTGTTTTATATAATGCATGTATTCACTAAAATAAAATTTAAAAAACGTCCTGTCTTGCTAGACAAGGTTGTGCATGTGCCTGTCACTACTGAGTCTGTCTACCTAT

GGATTTGCATTTTTGTATTTTGTACAAAGTAAAAATAACT
```

FIG. 2-2

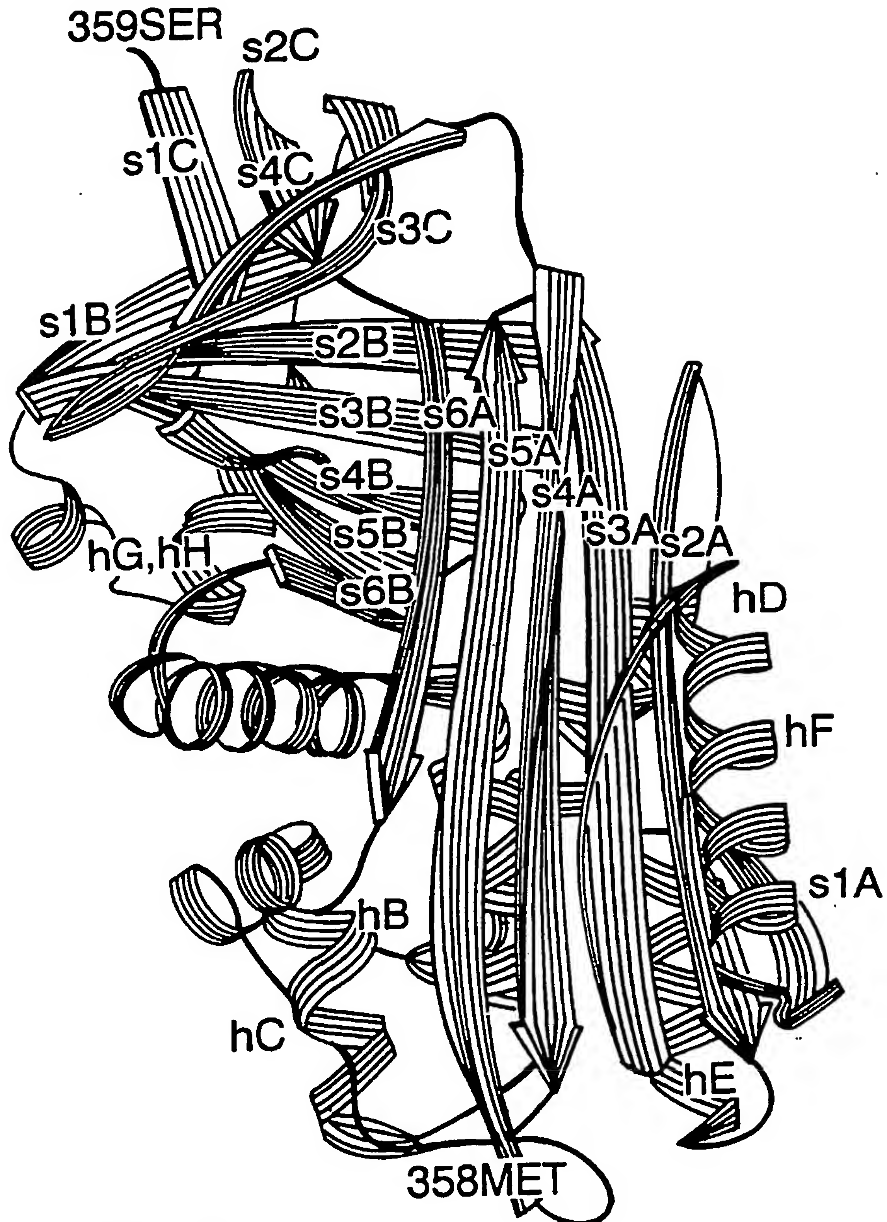
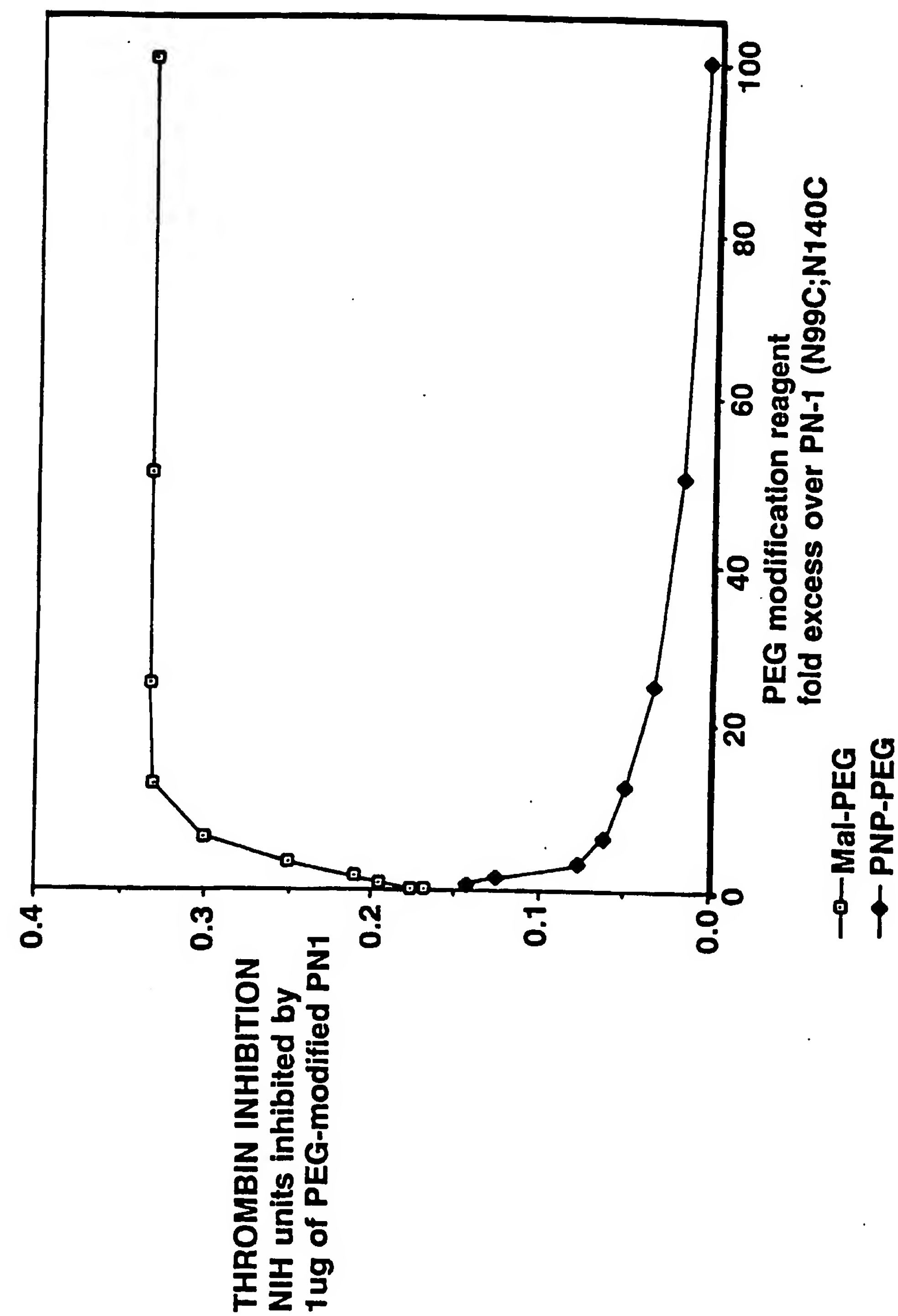

359SER
s2C
s1C
s4C
s3C
s1B
s2B
s3B
s6A
s5A
s4B
s4A
s3A
s2A
hG,hH
s5B
s6B
hD
hF
s1A
hB
hC
hE
358MET

FIG. 3



THROMBIN INHIBITION
NIH units inhibited by
1ug of PEG-modified PN1
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
PEG modification reagent
fold excess over PN-1 (N99C;N140C
Mal-PEG
PNP-PEG

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11624

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12P 21/06; C12N 15/00, 9/96; A61K 38/00
US CL : 435/69.2, 69.7, 172.1, 188; 530/345
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.2, 69.7, 172.1, 188; 530/345

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,166,322 (SHAW ET AL.) 24 November 1992, see entire document.	1-53
Y	US, A, 5,206,344 (KATRE ET AL.) 27 April 1993, see entire document.	1-53

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 FEBRUARY 1995	17 FEB 1995

Name and mailing address of the ISA/US	Authorized officer
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